OCEANOGRAPHIC CONTROLS OF HYDROCARBON DEGRADATION IN THE GULF OF MEXICO

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OCEANOGRAPHIC CONTROLS OF HYDROCARBON DEGRADATION IN THE GULF OF MEXIC

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LIST OF SYMBOLS AND ABBREVIATIONS

GOM	Gulf of Mexico
DWH	Deepwater Horizon
HC	Hydrocarbon
DNA	Deoxyribonucleic acid
RNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
RT-PCR	Revers transcription polymerase chain reaction
cDNA	Complementary DNA
SSU rRNA	Small subunit ribosomal ribonucleic acid
*nif*H	Nitrogenase subunit H
DOR	Dispersant to oil ratio
DCM	Dichloromethane
EPA	Environmental Protection Agency
OTU	Operational taxonomic unit
PAH	Polycyclic aromatic hydrocarbon
LMW	Low molecular weight
TPH	Total petroleum hydrocarbon
WAF	Water accommodated fraction
CEWAF	Chemical enhanced water accommodated fraction
PERMANOVA	Permutational analysis of variance
Ea	Activation energy
Q_{10}	Temperature coefficient

k	Pseudo first order rate coefficient
T_{opt}	Optimum temperature
OSCAR	Oil spill contingency and response model

SUMMARY

Petroleum is a critical industrial resource which can be refined into various important products. As the global demand for oil rises and traditional shallow marine oil reserves become depleted, exploration activities are moving towards offshore regions. In parallel, global warming has resulted in an elongated ice-free period in polar seas, which facilitates oil exploration in pristine, high-latitude regions. Exploration activities expose pristine offshore and polar ecosystems to an elevated risk of oil contamination, as evidenced by the Deepwater Horizon (DWH) oil spill. The DWH catastrophe represents the largest accidental marine oil spill in human history, which released 3.19 million barrel equivalents of crude oil into the Gulf of Mexico. Along with the released oil, 1.84 million gallons of dispersant were applied to both the wellhead and at the surface. The DWH discharge differed from most previous oil spills in that it occurred at the wellhead, which was 1,500 m below the sea surface. The trajectory of released oil was initially exposed to a combination of extreme environmental conditions, including high pressure and low temperature. Microbial biodegradation is considered to be the primary means by which spilled oil is eliminated from the environment. Although the controls of petroleum hydrocarbon biodegradation have been studied for years, there is as yet no consensus on the results for predictive modeling. Thus, the overall goal of this dissertation was to unravel how various oceanographic parameters control hydrocarbon biodegradation potential.

Specific objectives of the dissertation were to:

1. Quantify rates of petroleum hydrocarbon biodegradation in seawater and sediments under close to in situ conditions.

2. Investigate the interaction between various oceanographic parameters that limit biodegradation under conditions relevant to the DWH oil spill

3. Determine the fate of individual hydrocarbon compounds or compound classes under varying oceanographic or sedimentary conditions.

4. Link degradation pathways to microbial community dynamics and environmental functions.

Dispersant application is a primary emergency response strategy employed to minimize ecosystem damage after an oil spill. The efficacy of dispersant application remains controversial, largely due to a lack of studies conducted at close to in situ conditions. To address this knowledge gap, *ex situ* incubations were conducted to quantify the impact of dispersant on petroleum hydrocarbon (HC) biodegradation rates and microbial community structure at close to *in situ* conditions in surface seawater. The results revealed that dispersant application substantially enhanced oil degradation under well-mixed conditions in surface seawater from the Gulf of Mexico. The overall half-life of petroleum hydrocarbons was reduced by 43 %, from 15.4 days to 8.8 days, in dispersed oil treatments as compared to those amended with oil alone. While the abundance of overall bacteria remained constant in all incubations, the abundance of nitrogen-fixing bacteria, as determined by qPCR of nitrogenase (*nifH*) genes, showed a large increase at 15 to 22 days. Respiration and growth of microbial communities were neither inhibited nor enhanced by dispersant treatment. Using next generation sequencing of small subunit rRNA gene amplicons from RNA and DNA extracts, dispersant was shown to have a pronounced impact on the composition of metabolically-active microbial communities. While

hydrocarbon-degrading microbial groups were enriched in both oil and oil + dispersant treatments, community composition diverged between treatments as incubation time progressed and converged at the end of the incubation in parallel with hydrocarbon degradation. Although *Gammaproteobacteria* (*Alteromonadaceae, Oceanospirillaceae*) dominated in the early stages of all treatments, the latter stages of the incubations were predominated by the *Hyphomicrobiaceae* or members of the *Burkholderiales,* with and without dispersant treatment, respectively. The quantitative increase in nitrogen-fixing members of the microbial community suggests a selection pressure for nitrogen fixation, likely indicating the robust response of indigenous microbial communities to a readily biodegradable nitrogen-poor substrate. In order to improve model predictions and the bioremediation of dispersed oil during emergency response efforts, future study is warranted on the coupling of biodegradation to nitrogen fixation.

In undispersed surface oil slicks, previous studies present conflicting evidence as to whether hydrocarbon degradation potential is determined by temperature, nutrient limitation, or the indigenous microbial community. For this reason, hydrocarbon biodegradation is often considered to be site-specific and related to exposure history. In this dissertation, a comparison of samples from three geographical regions of major oil exploration (Beaufort Sea in the Arctic, northern and southern Gulf of Mexico, GOM) provided unexpected results. It was hypothesized that the GOM is primed for rapid hydrocarbon biodegradation due to chronic oil input from natural hydrocarbon seepage. Contrary to the hypothesis, biodegradation rates quantified in oil-amended seawater microcosms were nearly always more rapid at the Arctic site. Nutrient amendment stimulated microbial growth in all microcosms and microbial abundance was higher at

optimum temperature of microbial activity. While maxima in degradation rates were always in the mesophilic temperature range, the temperature response of both microbial activity and diversity was site-specific and provided evidence for adaptation to in situ temperature. Taxonomic diversity of microbial communities was generally elevated in nutrient-amended treatments within the in situ temperature range of each site, while diversity remained the same or was lower at temperatures deviating from in situ conditions. Beta diversity showed a strong selection of microbial communities by site. Microbial communities in all microcosms were dominated by the *Gammaproteobacteria*. Community composition was selected by site, temperature, and nutrient condition. *Acinetobacter* showed the highest relative abundance (7- 89 %) in all incubations at warmer temperatures (above 8 ˚C) in unamended treatments, suggesting its preference for mesophilic temperatures and low nutrients common to more temperate waters. At mesophilic temperatures in the nutrient-amended treatments, *Marinobacter* and *Alcanivorax* showed the highest relative abundance along with *Acinetobacter*. The genus *Colwellia* was only observed in microcosms from the Arctic site that were incubated at close to in situ temperature, corroborating previous work indicating that this group is psychrophilic. The results indicate that both biodegradation activity and microbial community composition are dictated most strongly by the site of origin followed by nutrient availability and temperature.

For blowouts such as the DWH disaster that occur in the deep ocean, the overlying seawater leads to high hydraulic pressure. The importance of pressure on the fate of hydrocarbons has been long recognized, and yet relatively few studies have considered this parameter. Through *ex situ* incubation of deepsea sediments and waters collected from the

GOM, high pressures equivalent to ambient conditions were shown to enhance hydrocarbon biodegradation, as determined by oxygen consumption, while rates were not affected in surface waters incubated at elevated pressure. In microcosms of deepsea waters and sediments, hydrocarbon degradation was accelerated by 20 to 80 % in incubations exposed to 10 MPa (equivalent to pressure at 1000 m water depth) in comparison to those exposed to atmospheric pressure. In microcosms of deepsea sediments, high pressure did not substantially impact the composition of indigenous microbial communities at either the DNA or RNA level, with only a few microbial groups responding to pressure treatment. The impact of pressure was more pronounced in bottom water samples, as decreases in alpha diversity were observed at both the DNA and RNA level. Members of the class *Gammaproteobacteria*, which contains many known hydrocarbon-degrading bacterial groups, dominated in all incubations. Disturbance by oil addition or incubation pressure impacted community composition, with a different subset of microbial populations responding to each treatment. The genus *Cycloclasticus,* C1-B045 of the family *Alteromonadaceae*, *Oleispira,* and an uncultured member of the *Oceanospirillaceae* were enriched in oil-amended treatments. In contrast, the marine methylotrophic group *Colwellia* was inhibited by oil addition. Similar trends were also observed with pressure treatment. An uncultured *Oceanospirillaceae* strain was enriched in DSH10 bottom water samples at 10 MPa. This strain shares 100 % sequence similarity with the dominant strain detected in the deep oil plumes generated during the DWH oil spill, while OTUs related to *Oleispira antarctica*, a closely related cultured representative of the DWH *Oceanospirillaceae*, was the dominant oil-degrader present in incubations at atmospheric pressure in DSH10 bottom water. The contrasting relative abundance of these two groups

suggests the importance of pressure as a strain-specific controlling factor of hydrocarbon degradation.

In conclusion, multiple oceanographic controls of hydrocarbon degradation potential were investigated in seawater and sediments from representative areas of major oil exploration. This dissertation delivers rate measurements that can be employed immediately to improve numerical models to predict the fate and transport of spilled oil in the oceans. In addition, the dissertation research provides critical insights into the complex interplay of oceanographic parameters that limit hydrocarbon degradation.

CHAPTER 1. INTRODUCTION

As petroleum reservoirs in temperate shallow regions of the world's oceans become depleted, oil exploration is moving towards non-traditional regions, such as the deepwater and ultra-deepwaters of the outer continental shelf (OCS) as well as the Arctic. In addition, due to global climate change and an elongated ice-free season, new routes of marine transportation are opening up through the Northwest Passage in the Arctic [1]. Increasing exploration and transportation activities expose pristine offshore and polar ecosystems to an elevated risk of oil contamination, as evidenced by the Deepwater Horizon (DWH) oil spill in the Gulf of Mexico (GOM). A systematic understanding of the rates and controls of hydrocarbon biodegradation as well as the response of indigenous marine microbial communities to oil contamination will be critical for assessing the environmental risks of oil exploration. Predictive models, such as the oil spill contingency and response model (OSCAR)[2] developed in Norway, are employed for the assessment of environmental risk. Model predictions are confounded by a lack of knowledge of the environmental controls of biodegradation.

The Deepwater Horizon (DWH) oil spill represents the largest accidental marine oil spill in world history. On April 20th, 2010, the DWH oil rig exploded and sank to the bottom two days later. The accident cost 11 lives and released 3.19 million barrels of crude oil [3]. The unique nature of the DWH disaster lies not only with its size, but also the location where it occurred [4]. Oil was released from a broken riser pipe at approximately 1.5 km below the sea surface, and emergency responders applied an unprecedented 1.84 million gallons of dispersant (COREXIT 9500A and COREXIT 9527) into the GOM,

both at the wellhead and to the surface slick, in order to keep the oil away from the sea surface and sensitive coastal ecosystems [5]. According to the Unified Area Command, released oil from the DWH was dispersed throughout the water column and sediments of the outer continental shelf in the northern Gulf of Mexico [6]. Approximately 10 % of the total released oil rose to the surface and formed slicks [7], 35 % formed dispersed oil plumes in the deepsea [8,9], and up to 47 % was trapped in sediments at the seafloor [10]. The trajectory of oil was exposed to a set of distinct ecosystems. Understanding of how oceanographic factors regulate hydrocarbon degradation potential is essential to predicting the fate and transport of released oil.

Microbially-mediated biodegradation is considered as the primary means by which spilled oil is eliminated from the environment [11]. Hydrocarbon degradation is catalyzed by respiration in the largely aerobic ocean water column. Thus, the process is limited by mixing energy, temperature, nutrient availability, pressure, and functional potential of the indigenous microbial community [11,12]. Although biodegradation has been studied for decades in the marine environment, there is as yet no consensus on the rates and controls of the process [13]. The focus of this dissertation research is to uncover the complex interplay of oceanographic parameters that limit hydrocarbon biodegradation potential in the world's oceans.

1.1 Oceanographic controls of hydrocarbon degradation: temperature, nutrient availability, pressure, and the composition of indigenous microbial communities

A range of environmental parameters determines the capacity and efficiency of microbial communities to degrade petroleum hydrocarbons in marine ecosystems [14].

Indeed, the same environmental pressures that impact pristine microbial food webs are in play during an oil spill including: temperature, pressure, oxygen and nutrient availability, and physical or chemical form of the oil [11,12,15]. Local environmental conditions of temperature and the availability of oxygen and nutrients, which have been shown to limit the rate and extent of hydrocarbon degradation or weathering, are determined by physical processes and the exchange of water masses in the oceans.

Temperature has long been recognized as a critical parameter that regulates hydrocarbon biodegradation [10,11]. Degradation activity was documented from subzero temperatures to more than 80 °C. At the extremes, previous studies have observed that microbes can degrade oil in sea ice at temperatures below the freezing point [1,16], while hyperthermophilic HC-degraders, whose optimum temperature for growth is higher than 80 °C, were previously isolated from oil reservoirs [17]. In the world's oceans, temperature varies among different geographical regions as well as at different water depths. In the surface GOM, seawater is warm perennially with annual average of approximately 25 °C [18]. However, the majority of open ocean habitats, including polar regions, are permanently cold, with temperatures below 4 °C [19]. Therefore, further research is needed to understand the controls of biodegradation under cold conditions.

Within the ambient temperature range of most marine ecosystems (0 °C to 40 °C), a positive correlation is often observed between hydrocarbon degradation rates and temperature. The temperature coefficient Q_{10}, which represents the increase in rates of activity with a corresponding 10 °C increase in temperature, is often used for estimating microbial temperature response. Previous studies report Q_{10} values that often range

between 2 and 3 [20]. Although most previous *ex situ* studies observed a positive correlation between hydrocarbon degradation rates and temperature [21–24], there are, however, exceptions. For example, Delille et al. observed no significant temperature impact on hydrocarbon degradation rates in Antarctic coastal seawater between 4 and 20 °C [25].

Temperature may also affect the mechanisms or pathways of degradation for different classes of HCs. Although alkane degradation rates often exceed rates of PAH degradation at all temperatures [21], this degradation pattern for the two groups could be temperature-dependent in some cases [26]. Temperature is also a selective force on microbial community composition and functional potential. For example, the genus *Colwellia* is often found in high abundance in permanently cold environments, such as the deepsea oil plumes generated during the DWH spill or oil contaminated Arctic surface seawater [27], while this group is generally not detected in the warmer surface seawater in the GOM [28,29].

Microorganisms that catalyze HC degradation coupled to respiration depend on the uptake of major nutrients (N, P), and therefore, nutrient limitation is important factor that governs degradation rates. Petroleum is replete in carbon, but depleted in nutrients [30]. At high levels of oil contamination, such as in an undispersed oil slick, available nutrients will become limiting, thereby inhibiting microbial productivity [13,31–33]. For this reason, fertilizers have been employed as a bioremediation strategy, especially in coastal ecosystems impacted by oil spills [34]. However, in some cases, nutrient application was not successful or resulted in unintended consequences to ecosystems [34]. For example, nutrient amendment may alter the composition of microbial food webs and/ or result in eutrophication [26,35,36].

The average depth of the world's oceans is approximately 3,800 m, and the deepsea represents the largest habitat for biota on earth [37]. As evidenced by the renowned "lunch box" experiment, where human food was preserved in a submarine that fell to the seafloor for an extended period, pressure has long been implicated as an important factor for the regulation of microbial activity [12,38]. The first study of the impacts of pressure on marine microorganisms was reported by Zobell and Johnson in 1949 [39]. The effects of pressure were determined for bacteria isolated from terrestrial and marine habitats, and strains from the deepsea shown to grow under extremely high pressure. Microorganisms that thrive under elevated pressure conditions were first termed "barophilic", and this was later revised to piezophilic by Yayanos [40].

In the deepsea, surface-derived piezosensitive or piezotolerant bacteria are believed to coexist with native piezophilic bacteria[38]. Grossart et al. observed that a gradual increase in pressure selected different microbial populations in *ex situ* incubations [41]. Surface-derived populations can be transported through the water column to the deepsea via natural organic aggregates [42]. In an incubation testing the pressure effect on the surface seawater, exposing surface-derived enzymes to elevated pressure was shown to inhibit enzyme activity at pressures above 1 MPa, and activity was reduced by 90 % at 10 MPa [43]. Similar results were obtained in many other studies [44,45]. In contrast, deepsea bacteria adapt to elevated pressure by modifying either lipid [46,47] and/or protein structures [48,49]. Adaptation to ambient pressure was implicated by the surprisingly rapid substrate incorporation and respiration observed in the deepsea [50]. Therefore, in order to achieve accurate measurements on deepsea microbial activity, *ex situ* incubations should be conducted at *in situ* condition [44].

Hydrocarbon degradation potential is expected to differ across geographical locations in the oceans, since environmental controls (temperature, nutrient condition, as well as indigenous bacteria communities) vary with the mixing and movement of ocean water masses. Previous studies suggested that hydrocarbon degradation potential is site-specific and correlated with hydrocarbon exposure history [51,52]. The GOM is a prolific hydrocarbon basin, which receives approximately 604,150 liters of oil per year thorough natural seeps alone [53,54]. Because of chronic hydrocarbon exposure, it has been hypothesized that indigenous microbial communities in the GOM are primed for HC degradation and rapidly respond to oil input [4,36,55]. However, to the best of the author's knowledge, there is little evidence to support this hypothesis [51].

After the DWH discharge, many studies focused on the microbial response in deepsea oil plumes and shorelines [8,56–59], while less is known about the response in oligotrophic surface seawater. As traditional shallow oil reservoirs in temperate regions become depleted, oil exploration is moving towards high latitude regions, including in the Beaufort Sea [60] . Moreover, due to global climate change, an elongated ice-free season promotes marine transportation through the Northwest Passage in the Arctic [1]. These phenomena may lead to an increased risk in the potential for an oil spill in fragile, polar ecosystems [61]. Unlike the GOM which spans subtropical to tropical climates, cold temperatures in the Arctic may alter the form/ solubility of oil and inhibit biodegradation [62], thereby increasing oil longevity in the environment. To assess environmental risks, the hydrocarbon degradation process was investigated in Arctic surface seawater since mid-20th century [16,27,63–65]. However, few studies have focused on the degradation potential and microbial community response in oil slicks, especially in the Arctic. A systematic

understanding of the rates and controls of hydrocarbon biodegradation as well as the microbial community response will be critical for assessing the environmental risks of oil exploration in polar regions.

1.2 The impact of dispersant application in surface seawater

Dispersants represent complex mixtures of chemical surfactants in a hydrocarbon solvent – the surfactants lower the interfacial tension between oil and water, breaking the oil into small droplets, with an increased surface-to-volume ratio and availability for biodegradation [66,67]. Although dispersant application is widely used as an emergency response strategy, the efficacy of this approach remains under debate [13,55].

Previous studies indicate that dispersant can enhance [22], have little effect [68], or diminish [69] the rate and extent of petroleum hydrocarbon (HC) degradation in seawater. The range in the enhancement of biodegradation by dispersant can be explained in part by differences in the design and approach of previous experiments [55,70]. Experiments must be conducted with concentrations of oil/dispersant resembling those expected during emergency response to an oil spill [65]. During the DWH discharge, oil concentrations varied from 0 to over 10,000 ppm in the surface seawater, and the concentration was rapidly diluted with distance from the wellhead [71]. In a survey conducted by NOAA immediately after the DWH oil spill, the majority of observations were at or below the low ppm level [72,73]. At these low ppm concentrations of oil, bioavailable nutrients and oxygen are unlikely to limit microbial activity [74]. In contrast, the majority of previous experiments quantified biodegradation in seawater incubations using oil concentrations which are orders of magnitude higher than those expected during active response (i.e.

125 – 2500 ppm [75], 867 ppm [76], 83 and 833 ppm [22], and 1400 ppm [69]). Using such high oil concentrations in a closed system may cause depletion of nutrients and artifacts which hinder biodegradation [65]. Further, the dispersion process depends on the oceanic mixing conditions, and lab incubations should reflect the mixing energy in the environment [70,77,78].

Another uncertainty of dispersant application is their impact on indigenous microbial communities [55]. Dispersants may impact microbial communities in a number of ways. Some groups of microorganisms prefer to attach to oil slicks. Members of the *Gammaproteobacteria* (including *Pseudoalteromonas, Pseudomonas, Vibrio, Acinetobacter,* and *Alteromonas*) were observed in heavily oiled slicks; while *Cyanobacteria* and *Alphaproteobacteria* were more abundant in less oiled surface sheens during the DWH spill [28]. Also, using pure cultures isolated from beach sands impacted by the DWH discharge, Overholt et al. observed that the HC degradation activity of certain strains such as *Acinetobacter* and *Alcanivorax* was inhibited by dispersant, while growth was not affected [79]. Moreover, dispersants comprise a labile carbon source that may stimulate the growth of certain microbial populations [69,80], and in some cases, dispersant application was suggested to select against the most effective hydrocarbon degraders [81]. The consensus is that the response of marine microbial communities to dispersants is species-specific and may alter their structure and function [79].

The objectives of this dissertation are to:

1. Quantify rates of petroleum hydrocarbon biodegradation in seawater and sediments under close to in situ conditions.

2. Investigate the interaction between various oceanographic parameters that limit biodegradation under conditions relevant to the DWH oil spill, including the application of dispersant chemicals as a response strategy.

3. Determine the fate of individual hydrocarbon compounds or compound classes under varying oceanographic or sedimentary conditions.

4. Link degradation pathways to microbial community dynamics and environmental functions.

CHAPTER 2. DISPERSANT ENHANCES HYDROCARBON DEGRADATION AND ALTERS THE STRUCTURE OF METABOLICALLY ACTIVE MICROBIAL COMMUNITIES IN SHALLOS SEAWATER FROM THE NORTHEASTERN GULF OF MEXICO

Xiaoxu Sun, Lena Chu, Elisa Mercando, Roger C Prince, Isabel Romero, David Hollander, and Joel E Kostka

2.1 Abstract

Dispersant application is a primary emergency oil spill response strategy and yet the efficacy and unintended consequences of this approach to marine ecosystems remain controversial. To address these uncertainties, *ex situ* incubations were conducted to quantify the impact of dispersant on petroleum hydrocarbon (HC) biodegradation rates and microbial community structure at close to *in situ* conditions in surface seawater. Seawater microcosms were designed to simulate mixing energy that represents the surface waters during the Deepwater Horizon discharge in the Gulf of Mexico and treatments were amended with weathered oil (2 ppm), with or without dispersant (Corexit EC9500A) at a dispersant to oil ratio (DOR) of 1:15. Microcosms were incubated for 40 days, sampled at regular intervals, and analyzed for hydrocarbon chemistry and microbial parameters. Mixing energy was shown to be a critical variable, as degradation was not substantially affected by dispersant application in a boom designed to simulate an oil slick. In contrast, under completely dispersed conditions, biodegradation was substantially enhanced, decreasing the overall half-life of total HC compounds by 43 %, from 15.4 days to 8.8 days. While the abundance of overall bacteria remained constant in all incubations, the

abundance of nitrogen-fixing bacteria, as determined by qPCR of nitrogenase (*nifH*) genes, showed a large increase at 15 to 22 days. Respiration and growth of microbial communities were neither inhibited nor enhanced by dispersant treatment. Using next generation sequencing of small subunit rRNA gene amplicons from RNA and DNA extracts, dispersant was shown to have a pronounced impact on the composition of metabolically active microbial communities. While hydrocarbon-degrading microbial groups were enriched in both oil and oil + dispersant treatments, community composition diverged between treatments as incubation time progressed and converged at the end of the incubation in parallel with HC degradation. Although *Gammaproteobacteria* (*Alteromonadaceae, Oceanospirillaceae*) dominated in the early stages of all treatments, the latter stages of the incubations were predominated by the *Hyphomicrobiaceae* or members of the *Burkholderiales*, with and without dispersant treatment, respectively. Our results indicate that dispersant application is an effective method to facilitate oil degradation when the system is sufficiently mixed, especially for recalcitrant high molecular weight compounds. Evidence for selection pressure for nitrogen fixation corresponds with rapid biodegradation in the presence of dispersants, and should be further explored for a deeper understanding of how spilled oil is removed from the environment as well as how to enhance the process.

2.2 Introduction

The Deepwater Horizon (DWH) oil discharge represents the largest accidental marine oil spill in history [71,82]. A total of 3.19 million barrels of crude oil along with an unprecedented 1.84 million gallons of dispersant, COREXIT 9500A and COREXIT 9527, were released into the Gulf of Mexico (GOM) [3]. Nearly 60 % of the dispersant was applied at the sea surface [4]. Dispersants are complex mixtures of chemical surfactants in a hydrocarbon solvent – the surfactants lower the interfacial tension between oil and water,

breaking the oil into small droplets, with an increased surface-to-volume ratio and availability for biodegradation [66,67].

Although biodegradation has been studied for decades in seawater, there is as yet no consensus on the rates and controls of the process [13]. The rate of biodegradation is limited by a range of environmental parameters including oxygen [12], temperature [83], nutrient availability [32], pressure [84,85], and microbial community composition [14]. Moreover, recent studies reveal that the concentration and form of oil used in experiments is a major confounding factor in our understanding of biodegradation [13,81]. The biodegradation rate of dispersed oil slows as the concentration of oil is increased and autochthonous nutrients become limiting at oil concentrations above 250 ppm [13]. Although dispersant application is widely used as an emergency response strategy, the efficacy of this approach remains under debate [13,55]. Previous studies indicate that dispersant can enhance [22], have little effect [68], or diminish [69] the rate and extent of petroleum hydrocarbons (HCs) degradation in seawater. The range in the enhancement of biodegradation by dispersant can be explained in part by differences in the design and approach of previous experiments [55,70]. Experiments must be conducted with concentrations of oil/dispersant resembling those expected during emergency response to an oil spill [65]. During the DWH discharge, oil concentrations varied from 0 to over 10,000 ppm in the surface seawater, and the concentration was rapidly diluted with distance from the wellhead [71]. In a survey conducted by NOAA immediately after the DWH oil spill, the majority of observations were at or below the low ppm level [72,73]. At these low ppm concentrations of oil, bioavailable nutrients and oxygen are unlikely to limit microbial activity [74]. In contrast, the majority of previous experiments quantified biodegradation in seawater incubations

using oil concentrations which are orders of magnitude higher than those expected during active response (i.e. 125 – 2500 ppm [75], 867 ppm [76], 83 and 833 ppm [22], and 1400 ppm [69]). Using such high oil concentrations in a closed system may cause depletion of nutrients and artifacts which hinder biodegradation [65]. Further, the dispersion process depends on the oceanic mixing conditions, and lab incubations should reflect the mixing energy in the environment [70,77,78].

Another uncertainty of dispersant application is the potential impacts on indigenous microbial communities [55]. Previous studies come to equivocal conclusions by suggesting that dispersants may impede or stimulate the growth of microbial populations [69,80,81].

The objectives of this study were to investigate the biodegradation of dispersed oil in relation to mixing energy as well as the impacts of dispersant on the dynamics of metabolically active microbial communities in coastal seawater [13]. Surface seawater was amended with artificially weathered Macondo surrogate oil and dispersant under close to *in situ* conditions. Petroleum hydrocarbon degradation and microbial communities were characterized using a close coupling of analytical chemistry and next generation sequencing techniques. Sequencing from extracted RNA was used as a proxy for the metabolically active microbial communities. The results point to the importance of mixing energy and alteration of metabolically active microbial communities following dispersant application. Our results under environmentally relevant conditions can be used to improve models that predict the fate of dispersed oil during future emergency response efforts.

2.3 Methods

2.3.1 Sample collection and experimental setup

Surface seawater (top 1 m) was collected from Pensacola beach, FL (30°19' N, 87°10' W) with carboys in June and August, 2016. The sampling site was described in a previous study [58]. Seawater was immediately transported to the lab and aerated overnight. Microcosms were conducted as previously described by Prince and Butler with modifications described below [70]. Two treatments were tested: seawater amended with weathered oil (oil treatment) or seawater amended with weathered oil and dispersant (oil + dispersant treatment) (COREXIT EC9500A; Nalco Environmental Solutions LLC, TX, USA). Surrogate MC252 oil was weathered by evaporation in the lab to approximately 20 % weight loss [70,86]. Two experiments were conducted under identical conditions, with the exception that mixing energy was varied. For all enrichments, 1.5 L of seawater was incubated in 2 L glass bottles. In oil treatments, 3 µl of oil was added to a floating boom to create a surface oil slick[70]. For the low-mixing oil + dispersant treatment, 3 µl of weathered oil was added to a floating boom, followed by 0.2 µl of dispersant. For the high-mixing oil + dispersant treatment, 3.2 µl of premixed oil/dispersant mixture was added to the bottles without booms. The mixture was prepared with a dispersant to oil ratio (DOR) of 1:15, similar to that recommended for emergency oil spill response [55,70,87]. Bottles were incubated unsealed in an incubator at 100 RPM and 25 ºC in the dark. Autoclaved Nanopure water was routinely added to compensate for evaporation. Duplicate samples were sacrificed for both nucleic acid and oil extraction at 0, 7, 15, 22, 30, and 40 days. Additional bottles were capped with rubber stoppers for quantification of respiration. Oxygen concentrations were measured by a Presens Microx 4 with PSt7 non-invasive sensor spots (Presens, Germany) adhered to the inside of the glass bottle. Carbon dioxide concentrations were measured by injecting 100 µl of headspace into a GC-FID equipped

with a methanizer (Shimadzu, Japan). Nutrient concentrations were monitored throughout the incubation period. Nitrite/nitrate, ammonia and soluble phosphate measurements were determined using established methods [88–90].

2.3.2 Extraction and analysis of hydrocarbons

At each time point, bottles were frozen at -20 ºC until extraction. The low-mixing scenario enrichments were extracted and analyzed according to Prince and Butler[70]. The high-mixing scenario samples were extracted as follows. A 10 µl aliquot of mixed standard was added to each bottle to correct for extraction efficiency. A 200 ml volume of 100 % ACS grade dichloromethane (DCM) (BDH Chemicals, UK) was added to each bottle and gently shaken for 30 seconds. The DCM layer was then carefully transferred to a separatory funnel and collected in a flask from the bottom. DCM treatment was repeated 3 times to recover all the residual oil from the bottle. The retrieved DCM layer was then reduced in volume to 5 ml in a Turbovap (Biotage, Uppsala, Sweden) followed by addition of 20 ml of 100 % ACS grade hexane (Fisher Chemical, New Jersey, USA). The volume of the extract was further reduced to 1 ml and stored in Brown glass HPLC vials at 4 ºC until analysis. Extracted oil samples from low-mixing enrichments were analyzed by GC-MS as previously described [70]. The high-mixing samples were analyzed based on previous literature with optimization[91,92]. Briefly, samples were analyzed on a GC/MS/MS with a quadrupole mass spectrometer (Agilent, California, USA), following EPA methods (Method 8270D). DWH crude oil (NIST 2779) was used as a reference standard.

Since HCs were extracted with different protocols and analyzed on different GC-MS instruments for the two experiments, results are reported as relative values to T0, with respect to 17α(H),21β(H)-hopane as a conserved internal standard[93]. Half-lives of each class of HCs were calculated using a first order kinetic equation.

2.3.3 Nucleic acid extraction and microbial community characterization

Only the high mixing scenario samples were extracted and analyzed for microbial community characterization. All 1.5 L of seawater from each bottle was filtered through 0.2 µm Mobio Powerwater Filter (MoBio Laboratories, CA, USA) and stored at -80 ºC before extraction. Each half of the filter was extracted for DNA and RNA using the Mobio Powerwater DNA isolation kit (MoBio Laboratories, CA, USA) and Mobio Powerwater RNA isolation kit (MoBio Laboratories, CA, USA), respectively, following the manufacturer's protocols. RNA was reverse-transcribed to cDNA using the qScript XLT cDNA supermix kit (Quantabio, MA, USA). Extracted DNA or cDNA was quantified with the Qubit HS assay kit (Invitrogen, Carlsbad, CA, USA) and 10 ng per reaction was used to generate SSU rRNA amplicons. Prokaryotic community composition was determined by applying a high-throughput sequencing-based protocol that targets PCR-generated amplicons from the V4 variable regions of the SSU rRNA gene using the primer set CS1_515F (5'-CACTGACGACATGGTTCTACA_GTGCCAGCMGCCGCGGTAA) and CS2_806R (5'-TACGGTAGCAGAGACTTGGTCT_GGACTACHVGGGTWTCTAAT) [94,95]. The resulting SSU rRNA gene amplicons were barcoded with unique 10-base barcodes (Fluidigm Corporation, CA, USA), pooled into equal DNA aliquots, and sequenced on an

Illumina MiSeq2000 platform at the DNA services facility of the University of Chicago according to established methods [96–98].

Sequence libraries were processed using multiple bioinformatics tools. Paired-end reads were merged with PEAR. The merged sequences were then demultiplexed and trimmed with vsearch and mothur, respectively. Chimeras were detected and removed with vsearch. Dereplicated sequences were clustered into OTUs using SWARM, with d = 1 [99]. Representative sequences were then taxonomically assigned via RDP classifier against the SILVA database [100,101].

Quantification of SSU rRNA genes and dinitrogenase genes was performed using quantitative PCR on a StepOnePlus platform (Applied Biosystems, CA, USA). The qPCR master mix were used the PowerUp SYBR Green Master Mix (Applied Biosystems, CA, USA). Quantification of 16S rRNA were used the primer set 331F(5'-CCTACGGGAGGCAGCAGT-3')/518R(5'-ATTACCGCGGCTGCTG-3')[102]. Standard curves were obtained with serial dilution of standard plasmids containing target Escherichia coli k12 16S rRNA as the insert (2.76×10^3 to 2.76×10^8 copy). The running condition was: 2 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 1 min. The quantification of the *nif*H gene used the primer set PolF(5'-TGCGAYCCSAARGCBGACTC-3')/PolR(5'-ATSGCCATCATYTCRCCGGA-3')[103]. Standard curves were obtained with serial dilution of standard plasmids containing target *Azotobacter vinelandii nif*H gene fragments as the insert (3.2×10^2 to 3.2×10^7 copy). The running condition was: 2 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 95 °C for 15 s, 63 °C for 1 min. In all experiments,

negative controls containing no template DNA were subjected to the same qPCR procedure to exclude or detect any possible DNA contamination.

2.4 Results and discussion

The primary objectives of this study were to investigate the influence of mixing energy on the ability of dispersant to enhance petroleum hydrocarbon (HC) biodegradation as well as to identify dispersant impacts on metabolically active microbial communities in surface seawater from the Gulf of Mexico. Boom treatments were used to simulate conditions when an oil slick is not treated with dispersant or under conditions when there is inadequate turbulence to mix dispersant with oil in the slick. The simulated oil slick remained in booms and was visible throughout the 40-day incubation with and without dispersant application. Treatments without booms represented a scenario, where the dispersant was efficiently mixed with oil by turbulence and thus biodegradation was further enhanced.

2.4.1 Impact of dispersant application on the rates and controls of hydrocarbon biodegradation

In the high mixing experiment, no significant differences in respiration rate were observed between treatments, with measured oxygen consumption rates of 4.39 ± 0.64 μmol O_2/L/day and 4.46 ± 0.75 μmol O_2/L/day for oil only and oil + dispersant treatments, respectively (Supplementary Figure 2-1). Similar to the oxygen consumption results, no difference was observed in CO_2 production rates between treatments (0.34 ± 0.01 μmol CO_2/L/day and 0.35 ± 0.02 μmol CO_2/L/day for oil only and oil + dispersant treatments respectively). In the low mixing experiment, a slight increase in respiration

rates was found in the dispersant treatment, but the difference was not statistically significant (2.71 ± 0.52 µmol O_2/L/day and 3.44 ± 0.52 µmol O_2/L/day for oil only and oil + dispersant treatments, respectively). The increase in CO_2 production was slightly increased from 0.41 ± 0.02 µmol CO_2/L/day to 0.49 ± 0.03 µmol CO_2/L/day when dispersant was applied. These results are corroborated by previous work conducted in seawater at low ppm oil concentrations, which showed no or minor change in oxygen consumption upon oil + dispersant treatment [19], and respiration rates from our study are comparable to those measured in the surface oil slick generated by the Deepwater Horizon (DWH) discharge (12.1 µmol O_2/L/day) [74]. The O_2 consumption to CO_2 production ratio was relatively high (~13). This could be explained by the formation of oxygenated compounds, which were unamenable by GC measurement [104]. Since, as we shall see below, dispersant plus mixing led to more biodegradation, these results indicate that oil biodegradation was only a small part of the indigenous respiration occurring in our incubations. In agreement, overall bacterial biomass, as determined by qPCR of SSU rRNA genes, was constant throughout all incubations. Further, major inorganic nutrients, inorganic nitrogen and phosphorus, remained relatively constant during the incubations (Supplementary Figure 2-2), again, similar to previous observations in experiments conducted at low concentrations of oil expected during emergency response efforts [19,74,105]. Nitrite/nitrate concentrations showed a slight increase during the late stages (after 22 days) of the incubations, perhaps due to the release of NH_4^+ during the mineralization of dead cells followed by nitrification to NO_3^- when labile carbon substrate availability decreased. Our observations indicate that overall respiration is neither inhibited nor enhanced by dispersant treatment, and dispersant does not appear to stimulate the total

growth of microbial communities at concentrations that resemble those expected during *in situ* response efforts. These findings agree with field observations during the DWH spill, where bacterial biomass remained constant within and outside the weathered surface oil slick [74]. In contrast, at similar oil concentrations, Wang et al. reported a 5-fold increase in microbial abundance during incubation of deeper waters (collected from 1500 m water depth) amended with dispersed oil[106]. Wang et al. may have observed growth due to the fact that their seawater mesocosms were amended with fresh crude oil along with dispersant, whereas our study employed weathered Macondo surrogate oil, which is more recalcitrant to biodegradation, as it contains a decreased portion of low molecular weight (LMW) compounds. Depletion of the labile portion of oil will reduce substrate quality and therefore diminish bacterial growth efficiency[107]. Alternatively, nutrient concentrations were an order of magnitude higher in the deep seawater used by Wang et al. which could have supported enhanced growth.

Our experiments were conducted with freshly collected seawater, and the active microbial community immediately responded to oil addition, with no observed lag phase in activity. In agreement with field and other laboratory observations, we found that low molecular weight hydrocarbons were degraded first followed by more recalcitrant compounds, especially multi-methylated PAHs (Figure 2-1) [108]. In all incubations, alkanes between C12 and C16 were degraded rapidly. The degradability of alkanes decreased with increasing chain length [8], but the ratio for n-C17/pristane and n-C18/phytane decreased with time in all treatments, clearly indicating that the loss of HCs was due to degradation [109]. Naphthalene and its homologues were rapidly degraded or volatilized within the first week of incubation in all treatments, whereas phenanthrene

and its homologues showed a high recalcitrance to biodegradation in all incubations other than the high mixing dispersant treatment.

The efficacy of dispersant treatment strongly depended on the mixing condition (Figure 2-1). In treatments with booms at the mixing energy used here, no difference was observed between treatments with or without dispersant, while the degradation rate was significantly elevated in the dispersant treatment in the high mixing scenario (without booms). This suggests that when directly applied to the oil slick without sufficient mixing, dispersant will not enhance hydrocarbon degradation. Under sufficient mixing energy, the application of dispersant enhanced the degradation of nearly all hydrocarbon groups. Half-lives of total petroleum hydrocarbons (TPH) were 15.4 days and 8.8 days for the oil only and oil + dispersant treatments, respectively (Supplementary Figure 2-3). These results are similar to previous measurements of 14 days for chemically dispersed oil in surface water [70] as well as degradation rates measured in simulated oil plumes [8,106,110]. In addition, our results are in agreement with model predictions of half-lives [111].

The effect of dispersant on biodegradation was more pronounced for high molecular weight compounds (Figure 2-1), including phenanthrene and its homologues and long chain alkanes ($C \geq 17$). Due to their low solubility, these compounds are likely to remain with droplets, and therefore dispersant application enhances degradation by decreasing droplet size and increasing the surface-to-volume ratio of the oil-water interface [13,112,113].

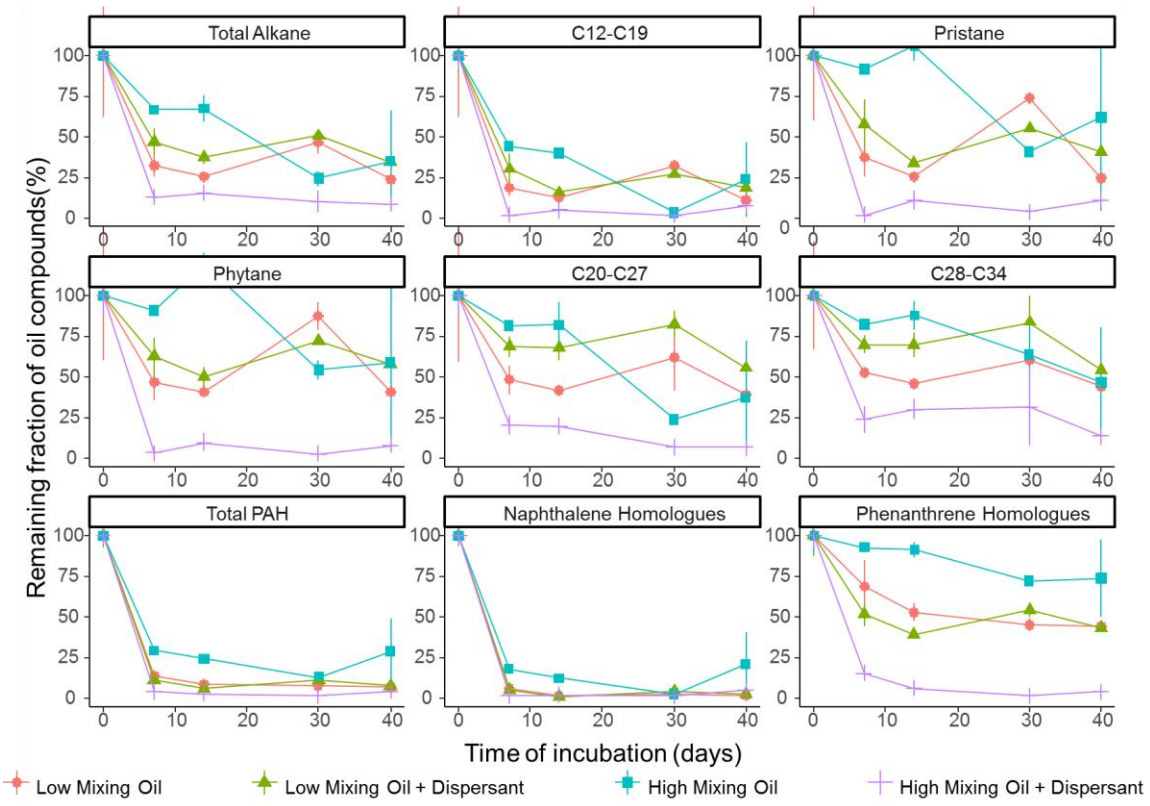


Figure 2-1. Degradation of petroleum hydrocarbons in microcosms of surface seawater. Hydrocarbon compounds are clustered based on their chemical properties and structure. Values shown are averages of duplicate measurements. Error bars are standard deviations.

Some earlier studies have observed no increase in hydrocarbon degradation after dispersant application [69,114] or concluded that microorganisms use dispersant as a carbon substrate and therefore attenuate hydrocarbon degradation [69,81]. Inconsistencies in the effects of dispersant on hydrocarbon degradation should be interpreted with caution and may be attributed to methodological considerations. Studies have often employed oil, nutrient amendments, or dispersant-to-oil ratios that are far from *in situ* conditions expected during response efforts after a spill [65]. Experiments that employ a water accommodated fraction (WAF) or a chemically enhanced water accommodated fraction (CEWAF) are difficult to compare to those in which oil and dispersant are directly added

to seawater. The WAF method excludes floating oil, which is likely to have a major impact on the activity of microbial communities in the incubations [115]. For example, using WAF and CEWAF treatments in seawater incubations, previous studies suggested that dispersant could suppress hydrocarbon degradation by selecting for dispersant degrading bacteria and against the most effective hydrocarbon degraders [81,116]. However, it is unclear what portion of the amended oil was contained in the incubations and it is possible that oil droplets that were not entrained during the production of WAF or CEWAF would be excluded and therefore alter the availability of oil for biodegradation [115]. A higher oil carbon content in the WAF treatment could explain why microbial activity was higher relative to that of the CEWAF treatment. When dispersant is applied to oil slicks at the ocean surface, undispersed oil will remain in the slick, potentially hindering biodegradation. Therefore, this study focused on the comparison of a simulated oil slick to fully dispersed oil [117]. The results confirm that dispersant application requires sufficient mixing power to disperse oil slicks [66,67]. With sufficient mixing energy, dispersant enhances hydrocarbon degradation. Other environmental parameters, such as oxygen and nutrient availability, are unlikely to limit biodegradation at low oil concentrations [65].

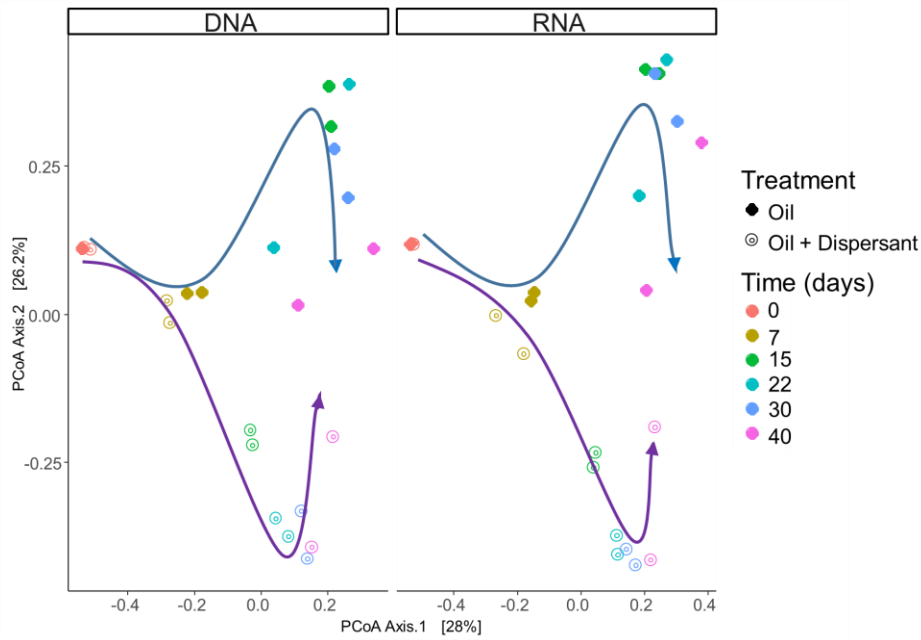


Figure 2-2. Comparison of microbial community composition with incubation time and treatment in seawater microcosms. Left and right panels show microbial community composition from sequencing of SSU rRNA genes in DNA and RNA extracts, respectively. Arrows highlight the changes in microbial community composition: blue arrows indicate oil treatments, purple arrows indicate the oil + dispersant treatments. Similarities between microbial communities are displayed as the Bray-Curtis distance metric on a PCoA plot.

2.4.2 Response of the metabolically active microbial communities to dispersant application

Microbial communities were characterized in high mixing energy treatments (without booms) that effectively mimic the dynamics of microbial populations that carry out biodegradation of completely dispersed oil in seawater. We used RNA sequences as a proxy to characterize the metabolically active microbial populations in oil-contaminated marine ecosystems. In general, very similar trends in community composition were observed over time at the RNA and DNA level (Figures 2-2 to 2-3), indicating that the majority of groups detected are metabolically active. According to PERMANOVA

analysis [118], both time and treatment were statistically significant factors that shaped microbial communities.

A succession of microbial populations, dominated by those affiliated with known hydrocarbon-degrading bacterial groups, was observed in parallel with the chemical evolution of petroleum hydrocarbon compounds (Figure 2-2). Thus, microbial succession is likely determined by the residual hydrocarbons present. Microbial community composition of the oil only and oil + dispersant treatments clearly diverged from day 15, suggesting dispersant acts as an additional selective force on microbial communities along with oil. By the end of the incubation (day 40), communities converged toward initial conditions, indicating community resilience and recovery from the initial disturbance Similar to many previous studies that have employed next generation sequencing technologies [112], a large fraction of the retrieved sequences could not be assigned at the genus level, indicating that most microbial populations remain uncharacterized. Low molecular weight alkanes and PAHs were degraded first (within 15 days) followed by recalcitrant higher molecular weight PAHs (more than 15 days). In parallel, microbial communities during the first two weeks in all treatments were dominated by the class *Gammaproteobacteria* (Supplementary Figure 2-4), which contributed up to 63 % (DNA) and 58 % (RNA) relative sequence abundance in oil only treatments, and 44 % (DNA) and 45 % (RNA) in dispersant treatments. The *Gammaproteobacteria* group contains many known. aerobic hydrocarbon-degrading bacteria, such as *Alcanivorax,* that were shown to rapidly respond to oil deposition in marine ecosystems[8,58,119–122]. For example, similar to our previous field observations in Pensacola beach sands that were impacted by oil from the DWH disaster, a bloom of

Alcanivorax was observed in the oil treatments of this study (Figure 2-3a) [58]. However, *Alcanivorax* was not observed in abundance in the dispersant treatments, which may indicate that Corexit 9500 inhibits the adherence and growth of *Alcanivorax,* as suggested in a previous study[123]. Alternatively, dispersant application may have dramatically enhanced alkane availability and resulted in such a rapid response of *Alcanivorax* that by our first sampling point, day 7, this group was already succeeded by other taxa.

Microbial groups shown to be capable of low molecular weight-PAH utilization were also enriched during the first 15 days of incubation. An abundant operational taxonomic unit (OTU) was observed that shows 98 % sequence identity to the type species *Polycyclovorans algicola* of the family *Solimonadaceae* (Figure 2-3a), a microbial group shown to utilize both aliphatic and PAH compounds [124,125]. The substrate range of this group would account for its presence throughout the incubations, including when labile alkanes and low molecular weight PAHs were depleted. *Polycyclovorans* was detected in all treatments, suggesting that it is insensitive to dispersant. In addition, members of the *Rhodobacteraceae* (Figure 2-3b), which contains known low molecular weight PAH degraders[126], were abundant in the early stages of the incubation, contributing up to 22 % and 24 % at the RNA level in oil and dispersant treatment at day 7, respectively.

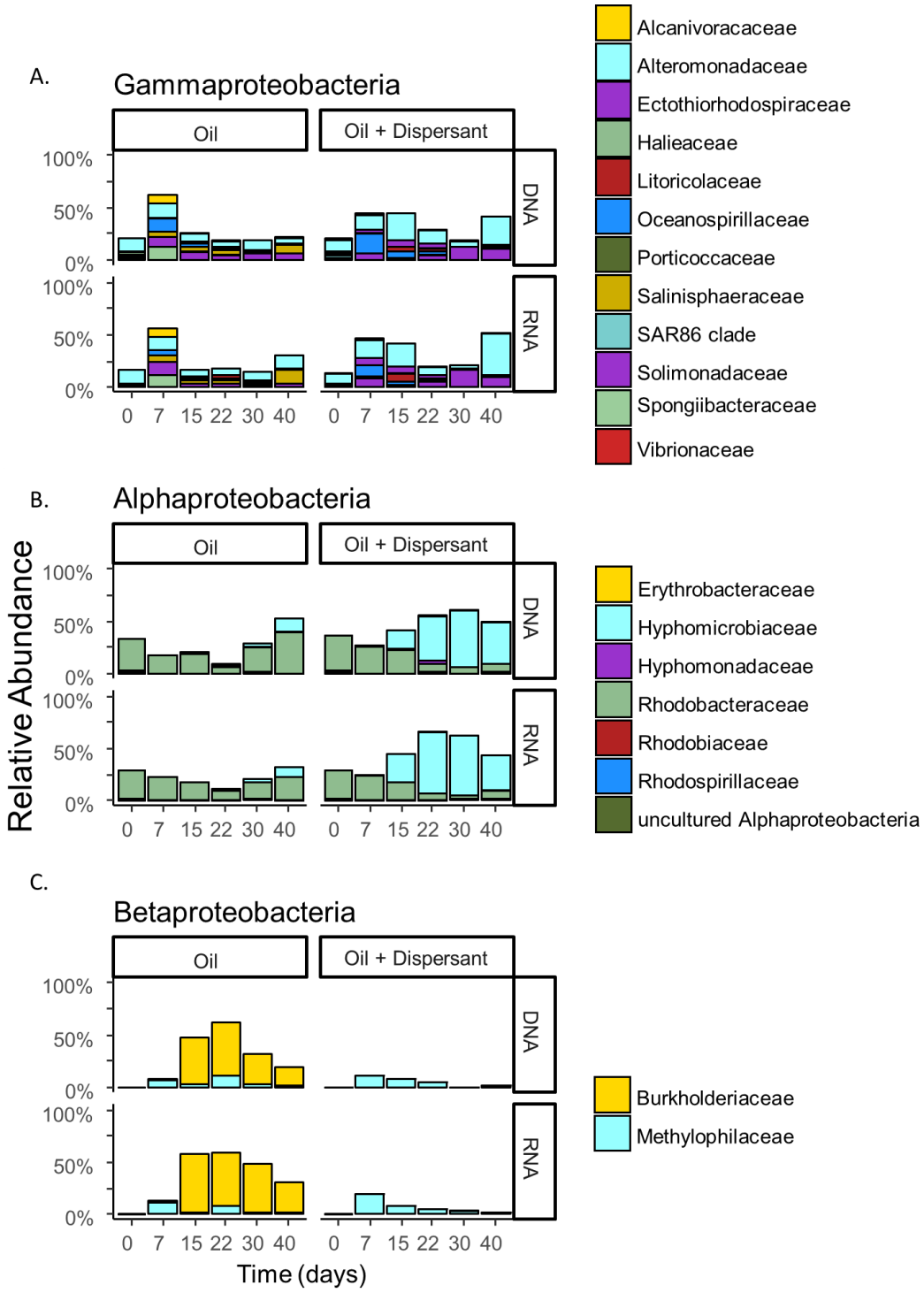


Figure 2-3. The relative abundance of (A) *Gammaproteobacteria*, (B) *Alphaproteobacteria*, and (C) *Betaproteobacteria* with incubation time and treatment. Barplots show mean value of duplicated samples. Taxa are grouped at the family level and relative abundance is calculated relative to total sequences.

Degradation occurred at a slower rate in the oil only treatment, in concurrence with a more prolonged bloom of microbial populations capable of degrading low molecular weight -PAHs. Members of the *Burkholderiaceae* within the *Betaproteobacteria* dominated in oil only treatments during the middle to late stages of incubation, comprising up to 51 % of the total DNA library on day 22 and 56 % of the total RNA library on day 15 (Figure 2-3c). The dominance of this group is noteworthy, as members of the *Betaproteobacteria* usually comprise a relatively low percentage of marine bacterioplankton. The most abundant OTU of this group was closely related (97 % similarity) to *Limnobacter litoralis* KP1-19 [127]; previous work detected the essential genes for aromatic compound degradation in *Limnobacter* [128], and this microbial group responded to crude oil addition in concurrence with the presence of PAHs [129]. The absence of this group in the dispersant treatment may suggest its growth was inhibited by the presence of dispersed oil or an inability to compete with other PAH degraders in dispersed oil.

After depletion of all low molecular weight hydrocarbons, two major families of the *Alphaproteobacteria* increased in relative abundance (Figure 2-3b). In the oil only treatments, several groups within the *Rhodobacteraceae* dominated during the latter stages of incubation, in agreement with previous observations attributing the degradation of more recalcitrant hydrocarbons to members of the *Rhodobacteraceae* [58]. The dominant OTU showed high sequence similarity to *Marivita roseacus* (99 %) of the *Roseobacter* lineage [130]. This lineage is a ubiquitous marine bacterial group that associates with phytoplankton cells or organic particles in the surface ocean [131]. This cell-surface interaction may explain the abundance of this lineage in the oil treatment,

possibly attaching to particles in the oil slick, while the absence of slick in the dispersant treatment would be less favorable. Some phytoplankton-associated microorganisms utilize hydrocarbons, both alkanes and PAHs, as they grow on natural hydrocarbons produced or accumulated by algae [126,132]. One recent study suggests that members of the *Roseobacter* group have the potential to degrade aromatic hydrocarbons[133]. Thompson et al also found members of this *lineage* were enriched by oil, especially in the presence of phytoplankton [134].

An OTU within the family *Hyphomicrobiaceae* dominated the microbial community from day 15 in the oil + dispersant treatment, constituting up to 55 % and 57 % at the DNA and RNA level, respectively (Figure 2-3b). A bloom of *Hyphomicrobiaceae* was observed at day 15, when both naphthalene and phenanthrene homologues were depleted in the oil + dispersant treatment. Further, an abundance of this group was also observed in the oil treatment at day 40, which suggests a similar community response to reduced availability of low molecular weight hydrocarbons. In corroboration of our results, members of the *Hyphomicrobiaceae* were demonstrated to degrade hydrocarbons in pure culture and were enriched in previous studies of oil contaminated sites [135–137]. For example, Rodrigues et. al. found members from this group were associated with both fluoranthene and pyrene addition, indicating their ability to degrade more recalcitrant high molecular weight PAHs [29].

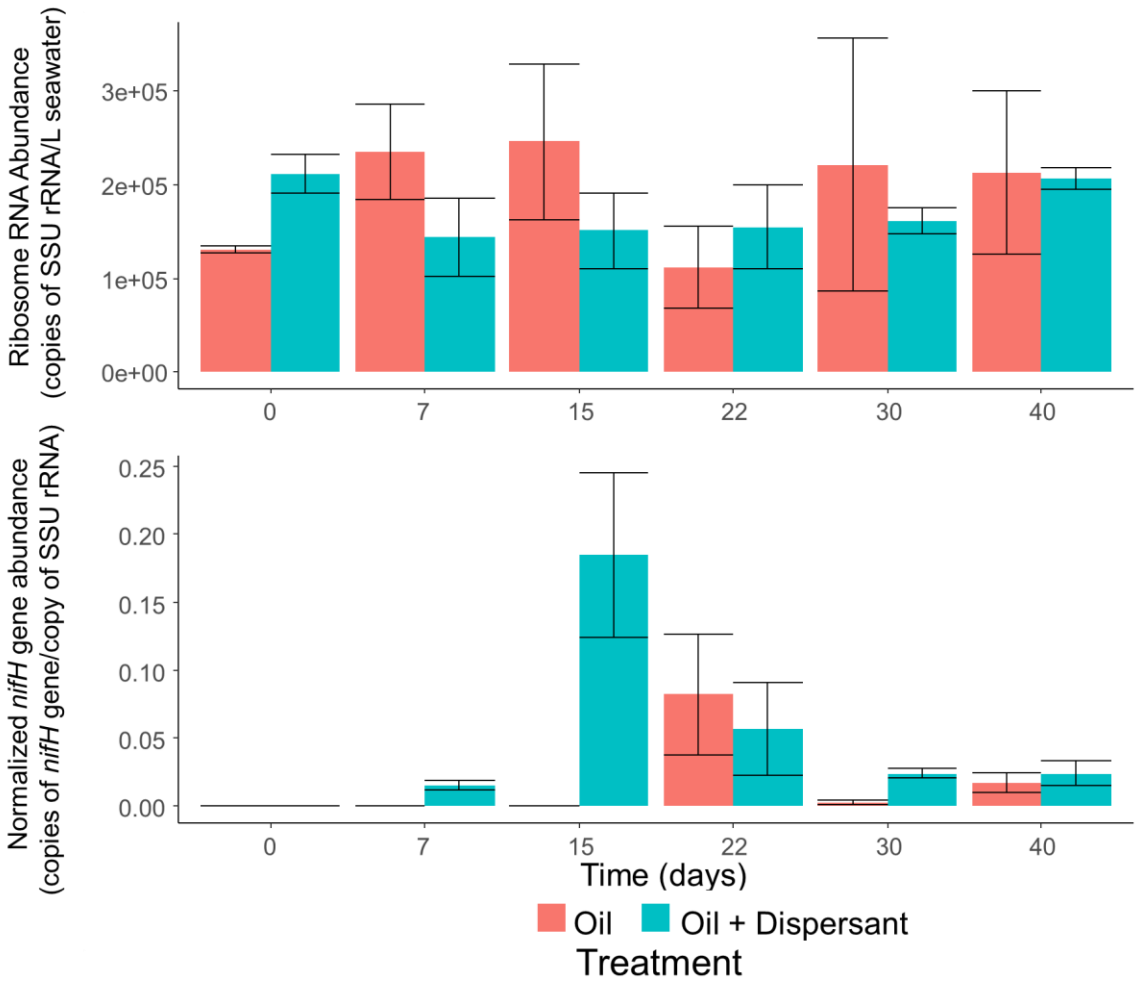


Figure 2-4. The abundance of overall bacteria and nitrogen-fixing prokaryotes in seawater microcosms as determined by quantitative PCR of a) SSU rRNA genes and b) *nifH* genes normalized to SSU rRNA genes. Error bars represent the standard deviation of duplicates.

Our results indicate that dispersant application did not alter the respiration or growth of microbial communities in seawater; or overall bacterial biomass, as determined by qPCR of SSU rRNA genes was constant throughout all incubations. Similar observations were made in the surface oil slick after the DWH spill [74]. In contrast, our qPCR results suggest that certain ecosystem functions are impacted by oil and dispersant treatment. The relative abundance of a key nitrogenase gene (*nifH*), which is a widely used molecular marker of

nitrogen fixation [138], was up-regulated during the middle stages of incubation (Fig 2-4b), and the maximum abundance of *nifH* in the oil + dispersant treatments was over twice that in the oil only treatments. The abundance of *nifH* in the oil + dispersant treatments peaked on day 15, comprising 18 % of the community as estimated by normalizing to the abundance of SSU rRNA genes. In the oil only treatments, a maximum abundance of 8 % was observed at day 22. The nitrogen fixation process was followed by the nitrification mentioned earlier. Neither nitrification nor nitrogen fixation process were able to associate to specific OTUs. Our results provide quantitative evidence that indicates microbial communities undergo nitrogen limitation in response to oil contamination in coastal ecosystems [139].

2.5 Conclusions

The results of this study indicate that when applying dispersant to an oil slick, biodegradation may not be substantially enhanced unless sufficient mixing energy is provided. When the simulated slick was sufficiently dispersed, a higher rate of removal was observed for more recalcitrant hydrocarbon compounds (such as phenanthrene), suggesting that surface area available for microbial colonization is a primary factor limiting hydrocarbon degradation, and the application of dispersant will likely alleviate this constraint. While microbial growth and respiration were not substantially altered, RNA analysis revealed that dispersant application resulted in pronounced changes to the composition of metabolically active microbial communities. The quantitative increase in nitrogen-fixing members of the microbial community suggests a selection pressure for nitrogen fixation, likely indicating the robust response of the indigenous microbial communities to a readily biodegradable nitrogen-poor substrate. In order to improve model

predictions and the bioremediation of dispersed oil during emergency response efforts, future study is warranted on the coupling of biodegradation to nitrogen fixation.

CHAPTER 3. HYDROCARBON BIODEGRADATION POTENTIAL IS SITE-SPECIFIC AND DETERMINED BY SYNERGIES BETWEEN TEMPERATURE AND NUTRIENT LIMITATION IN SURFACE OCEAN WATERS

Xiaoxu Sun and Joel E Kostka

3.1 Abstract

The risk of an oil spill accident is increasing in pristine regions of the world's oceans due to the development and transport of crude oil resources, especially in the Arctic as a result of the opening up of ice-free transportation routes. Although the controls of petroleum hydrocarbon biodegradation have been studied in surface ocean waters for years, there is as yet no consensus on the results for predictive modeling. Thus, the objective of this study was to quantify the potential for hydrocarbon biodegradation in surface waters of three sites that represent geographical regions of major oil exploration (Beaufort Sea in the Arctic, northern and southern Gulf of Mexico, GOM) in a systematic experimental design that incorporates gradients in temperature and the availability of major nutrients. Surface seawater was amended in microcosms with Macondo surrogate oil to simulate an oil slick, and microcosms were incubated with or without nutrient amendment at temperatures ranging from 4 to 38 $^{\circ}$C for 15 days. We hypothesized that GOM waters are primed for hydrocarbon biodegradation and nutrient availability overrides temperature as a biodegradation control.

Contrary to the hypothesis, biodegradation rates were nearly always more rapid at the Arctic site in comparison to the GOM sites. Nutrient amendment stimulated microbial growth in all microcosms and microbial abundance was higher at optimum temperature of microbial activity. While maxima in degradation rates were always in the mesophilic temperature range, the temperature response of both microbial activity and diversity was site-specific and provided evidence for adaptation to in situ temperature. Taxonomic diversity of microbial communities was generally elevated in nutrient amended treatments within the in situ temperature range of each site, while diversity remained the same or was lower at temperatures deviating from in situ conditions. Beta diversity showed a strong selection of microbial communities by site. Microbial communities in all microcosms were dominated by the *Gammaproteobacteria*. Community composition was selected by site, temperature, and nutrient condition. *Acinetobacter* showed the highest relative abundance (7-89 %) in all incubations at warmer temperatures (above 8 $^{\circ}$C) in unamended treatments, suggesting its preference for mesophilic temperatures and low nutrients common to more temperate waters. At mesophilic temperatures in the nutrient-amended treatments, *Marinobacter* and *Alcanivorax* showed the highest relative abundance along with *Acinetobacter*. The genus *Colwellia* was only observed in microcosms from the Arctic site that were incubated at close to in situ temperature, corroborating previous work indicating that this group is psychrophilic. Our results indicate that microbial adaptation to *in situ* conditions largely determines the hydrocarbon degradation potential.

3.2 Introduction

The Deepwater Horizon (DWH) catastrophe represents the largest accidental marine oil spill in human history [71,82]. Approximately 3.19 million barrel equivalents of oil were

released into the Gulf of Mexico (GOM) [3], and an estimated 10 % of the released oil formed slicks on the surface ocean [7]. While the majority of oil was rapidly dispersed, a persistent oil slick was present at the wellhead during and after the spill [140]. From June to November, 2010, the integrated surface oil concentrations in the northern GOM varied from non-detectable to over 10,000 ppm, with the highest concentrations observed near the wellhead and in coastal areas [71].

Microbial biodegradation is considered to be the primary means by which spilled oil is eliminated from the environment [11]. The potential for petroleum hydrocarbon (HC) degradation was previously shown to be site-specific and correlated with its hydrocarbon exposure history [51,52]. The GOM is a prolific hydrocarbon basin, which receives approximately 604,150 liters of oil per year thorough natural seeps alone [53,54]. Because of the chronic hydrocarbon exposure, it has been suggested that microbial communities in the GOM are primed for hydrocarbon degradation and could rapidly respond to oil input [4,36,55]. However, little evidence is available to support this hypothesis [141].

Hydrocarbon biodegradation is coupled to aerobic respiration in oceanic surface waters [15]. Like all respiration processes, biodegradation is limited by the availability of oxygen, nutrients, temperature, and the physiology of hydrocarbon-degrading microorganisms. Since HCs are often distributed in seawater as liquid droplets, the form and solubility of oil also limits biodegradation. A large body of research, including laboratory and field studies, has shown that the ocean environment dictates the efficiency and capacity of microbial communities to degrade hydrocarbons [36,142,143]. However, despite this extensive knowledge base, a quantitative understanding is lacking, and we have yet to determine how environmental factors interact to regulate the fate and transport of spilled

oil in the oceans. After the DWH disaster, many studies focused on the microbial response to oil contamination in deepsea oil plumes and on shorelines [8,56–59]. Less information is available on the microbial response to oil slicks in oligotrophic surface seawater

As traditional shallow oil reservoirs in temperate regions are depleting, oil exploration is moving towards high latitude regions, including in the Beaufort Sea [60] . Moreover, due to global climate change, an elongated ice-free season promotes marine transportation through the Northwest Passage [1]. Both of these phenomena may lead to an increased risk in the potential for an oil spill in fragile, polar ecosystems [61]. Unlike the GOM which spans subtropical to tropical climates, cold temperatures in the Arctic may alter the form/ solubility of oil and inhibit biodegradation [62], thereby increasing oil longevity in the environment. A systematic understanding of the rates and controls of hydrocarbon biodegradation as well as the microbial community response will be critical for assessing the environmental risks of oil exploration in polar-regions.

The majority of previous studies have focused on the degradation potential of dispersed oil in seawater, and the microbial community response in oil slicks at the surface remains understudied, especially under permanently cold conditions found in the Arctic. Thus, the objective of this study was to quantify the potential for hydrocarbon biodegradation in simulated oil slicks generated from the surface waters of three sites that represent geographical regions of major oil exploration (Beaufort Sea in the Arctic, northern and southern Gulf of Mexico, GOM) in a systematic experimental design that incorporates gradients in temperature and the availability of major nutrients. Surface seawater was amended in microcosms with Macondo surrogate oil to simulate an oil slick, and microcosms were incubated with or without nutrient amendment at temperatures

ranging from 4 to 38 ˚C for 15 days. Respiration rates served as a proxy for hydrocarbon degradation potential. Microbial abundance and community composition were determined by qPCR and next generation sequencing of SSU rRNA genes, respectively. The conclusions indicate that the site of origin may override nutrient availability and temperature in dictating hydrocarbon degradation potential. In addition, our results call into question the role of chronic oil pollution in the "priming" Gulf of Mexico waters for oil biodegradation.

3.3 Method

3.3.1 Sample collection

Surface water samples were collected in Niskin bottles at 10 m below the sea surface, from separate research cruises aboard the Canadian Coast Guard icebreaker Amundsen (September, 2015), R/V Weatherbird (August, 2015), and R/V Justo Sierra (July, 2015) for the polar site CB2, subtropical site DWH01, and tropical site IXTOC01, respectively. Site characteristics are presented in Table 1. Samples for nutrient analysis were immediately filtered through 0.2 µm polycarbonate filters (MoBio Laboratories, CA, USA) and stored at – 20 °C. Samples for incubation were stored in Nalgene bottles at 4 °C until use. Major inorganic nutrients (nitrite/nitrate, ammonia and soluble phosphate) were determined using established methods [88–90].

3.3.2 Microcosm experiments

For each site, microcosms were constructed by amending 5 ml of seawater with 5 µl of surrogate MC252 oil [86] in 30 ml sealed glass serum bottles. Experimental treatments

included: unamended (UN) microcosms to which no nutrient was added and nutrient amended (NA) microcosms that received 32 μM ammonium and 2 μM phosphate (final concentration) [74]. Microcosms with no oil addition were constructed and incubated at 25 °C to indicate the amount of respiration supported by natural organic matter present in seawater at the time of sampling. Triplicate microcosms were incubated in the dark at 6 different temperatures spanning the temperature range of polar to tropical climates (4, 8, 19, 25, 30, and 38 °C) for 15 days. Microcosms were sampled at regular intervals for respiration rate measurements.

Respiration was used as a proxy for oil biodegradation as established in previous work [57]. Respiration rates were quantified as CO_2 accumulation by sampling microcosm headspace with a gas-tight syringe and immediately injecting the sample into a GC-FID equipped with a methanizer (Shimadzu Scientific, Kyoto, Japan). Total carbon dioxide production was calculated as the sum of the gas phase and dissolved phase using Henry's law with temperature compensation [144]. Maximum respiration rates were calculated using the R package 'grofit' with default settings [145]. Calculation of activation energy (E_a) and temperature coefficient (Q_{10}) were carried out following the methods described in Bagi et al.[23]. Rate coefficients (k) were calculated using the pseudo-first order equation $\frac{dC}{dt} = -kt$, where C is residual carbon concentration (μM) and t is incubation time (days).

3.3.3 DNA extraction and sequence analysis

After incubation, all of the seawater volume was pelleted down and extracted with the Quick-DNA Fungal/Bacterial Microprep Kit (ZYMO Research, Irvine, CA). Extracted DNA was quantified with the Qubit HS assay kit (Invitrogen, Carlsbad, CA,

USA) and 10 ng per reaction was used to generate SSU rRNA amplicons. Prokaryotic community composition was determined by applying a high-throughput sequencing-based protocol that targets PCR-generated amplicons from the V4 variable regions of the SSU rRNA gene using the primer set CS1_515F (5'-ACACTGACGACATGGTTCTACA_GTGCCAGCMGCCGCGGTAA) and CS2_806R (5′-TACGGTAGCAGAGACTTGGTCT_GGACTACHVGGGTWTCTAAT)[94,95]. The resulting SSU rRNA gene amplicons were barcoded with unique 10-base barcodes (Fluidigm Corporation, CA, USA), pooled into equal DNA aliquots, and sequenced on an Illumina MiSeq2000 platform at the DNA services facility of the University of Chicago according to established methods [96–98].

Sequence libraries were processed using multiple bioinformatics tools. Paired-end reads were merged with PEAR. The merged sequences were then demultiplexed and trimmed with vsearch and mothur, respectively. Chimeras were detected and removed with vsearch. Dereplicated sequences were clustered into OTUs using SWARM, with d = 1 [99]. Representative sequences were then taxonomically assigned via RDP classifier against the SILVA database [100,101]. Downstream analysis were conducted in R using packages, including phyloseq, vegan, DESeq2, and ggplot2 [146]. PERMANOVA analysis was performed for statistical comparison of microbial community dissimilarity.

Quantification of SSU rRNA and nitrogen fixation genes (dinitrogenase, nifH) was performed using quantitative PCR on a StepOnePlus platform (Applied Biosystems, CA, USA). The PowerUp SYBR Green Master Mix (Applied Biosystems, CA, USA) was employed. Quantification of SSU rRNA genes used the primer set 331F(5'-

CCTACGGGAGGCAGCAGT-3')/518R(5'-ATTACCGCGGCTGCTG-3') [102]. Standard curves were obtained with serial dilution of standard plasmids containing target Escherichia coli k12 SSU rRNA as the insert (2.76×10^3 to 2.76×10^8 copy). Experimental conditions were set to: 2 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 1 min. Quantification of the *nifH* gene used the primer set PolF(5'-TGCGAYCCSAARGCBGACTC-3') /PolR(5'-ATSGCCATCATYTCRCCGGA-3')[103]. Standard curves were obtained with serial dilution of standard plasmids containing target *Azotobacter vinelandii nifH* gene fragments as the insert (3.2×10^2 to 3.2×10^7 copy). Experimental conditions were set to: 2 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 95 °C for 15 s, 63 °C for 1 min. In all experiments, negative controls containing no template DNA were subjected to the same qPCR procedure to exclude or detect any possible DNA contamination.

3.4 Results

3.4.1 Site characteristics

In situ nutrient concentrations and temperatures are provided in Table 1. As expected, temperature at the time of sampling was highest at the tropical site (IXTOC01), intermediate at the subtropical site (DWH01), and much lower at the polar site (CB2). Major nutrient concentrations, total inorganic nitrogen and soluble phosphorus, were highest at CB2 but these differences were not statistically significant ($p = 0.23$ for total nitrogen and $p = 0.18$ for phosphorus) (Table 1).

Table 3-1 Characteristics and results for each sampling site.

		CB2	DWH01	IXTOC01
Latitude		**75° 47 N**	**28° 43 N**	**19° 22 N**
Longitude		**129° 17 W**	**88° 23 W**	**92° 19 W**
Total Inorganic Nitrogen (µM)		**6.16 ± 3.0**	**2.83 ± 3.8**	**2.54 ± 2.1**
Phosphate (µM)		**2.08 ± 1.5**	**0.7 ± 0.1**	**1.72 ± 0.8**
In situ temperature (°C)		**0.7**	**31**	**35.9**
Optimum temperature (°C)		**30**	**38**	**38**
Activation Energy (kJ/mol)		**54.7 ± 11.3**	**83.1± 16.5**	**76.2 ± 12.0**
Q_{10} at 20 °C		**2.1**	**3.1**	**2.8**
Rate Constant at optimum temperature, k (day^{-1})	Unamended	**0.003 ± 0.0004**	**0.001 ± 0.0002**	**0.002 ± 0.0002**
	Nutrient Amended	**0.01 ± 0.002**	**0.008 ± 0.0006**	**0.009 ± 0.0002**
R_{opt} /$R_{4°C}$		**4**	**50**	**15**

3.4.2 Biodegradation rates

The temperature response of microbial respiration was determined in triplicate microcosms with and without nutrient amendment for all sites. Microbial respiration, as determined by CO_2 accumulation, was used as a proxy for hydrocarbon degradation, as in previous studies (Figure 3-1) [23,35,74,147]. This assumption is supported by the fact that little respiration was detected in control microcosms to which no oil was added. In addition, known hydrocarbon-degrading bacteria dominated the seawater microbial communities in all oil-amended treatments.

Whereas no oil controls showed little to no activity during the incubation period (Supplementary Figure 3-2), respiration began immediately in the remaining microcosm treatments and no significant lag phase was observed. Respiration rates were nearly always higher at CB2 in all treatments (236.4 ± 44.2 µmol CO_2/L/day for NA treatment and 42.1 ± 11.6 µmol CO_2/L/day for UN treatment) in comparison to the Gulf sites, except at the highest temperature studied, which is well above the ambient range for this permanently cold polar site (38 °C). Maximum rates at the GOM sites were 155.7 ± 29.0 µmol/L/day and 167.2 ± 8.1 µmol/L/day for NA treatment in DWH01 and IXTOC01 microcosms, respectively. Rates in the UN treatments were 11.5 ± 7.6 µmol CO_2/L/day and 18.7 ± 8.8 µmol CO_2/L/day for DWH01 and IXTOC01 microcosms, respectively. At the optimum temperature of activity determined for each site, rates at the GOM sites were 29 – 33 % and 16 – 30 % lower in comparison to CB2 rates in the NA and UN treatments, respectively.

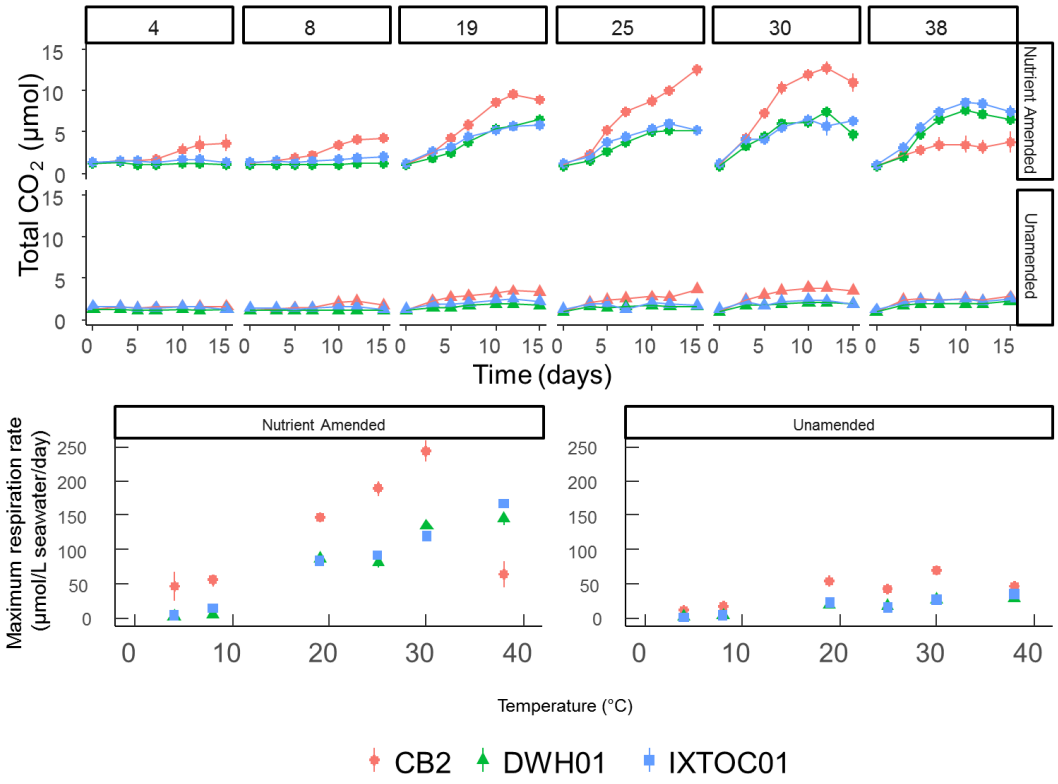


Figure 3-1. Carbon dioxide accumulation (upper panel) and estimated maximum respiration rates (lower panel). Scatter plots show average values from triplicate measurements. Error bars are standard deviations.

According to PERMANOVA analysis, nutrient amendment explained the largest amount of variation in respiration (38 %, p = 0.01) followed by temperature (12 %, p = 0.03), and site (7 %, p = 0.064) (Figure 3-1b). At the majority of temperatures studied, activity was stimulated substantially by nutrient amendment, with an average of 8.5 times increase in respiration rates. Interestingly, at cold temperatures well below the ambient range, activities were suppressed at the GOM sites and the addition of nutrients did not enhance degradation rate. Rates increased with incubation temperature, and showed maxima in the mesophilic range (at 30 °C for CB2 and 38 °C for the GOM samples). Our

study may not have captured the optimum temperature (T_{opt}) of biodegradation for the GOM sites as rates continued to increase throughout the range studied.

3.4.3 Microbial community analysis

Overall bacterial abundance was determined at the end of each incubation using qPCR of SSU rRNA genes (Supplementary Figure 3-1). Nutrient amendment apparently stimulated microbial growth in all mesocosms as bacterial abundance was 5 to 10 times higher in the NA treatments in comparison to the UN treatments. A positive correlation was observed from the linear regression between bacterial abundance and respiration rates at the T_{opt} ($R^2 = 0.44$, $p < 0.001$). For the GOM sites, bacterial abundance was much higher at the optimum temperature of microbial activity (30-38 °C) in comparison to the lowest temperature studied (4 °C). Although lower, bacterial abundance at 4 °C in the CB2 incubations comprised approximately half of that determined at the optimum temperature of activity. Neither temperature nor nutrient availability significantly affected the cell specific respiration rates.

Over 3 million paired-end reads were generated on an Illumina Miseq platform and 2.4 million SSU rRNA gene sequences remained after quality control. OTUs with a relative abundance of less than 0.05 % of the total reads, were removed. Samples that contained fewer than 6000 reads were also discarded.

Beta diversity, Bray-Curtis distance on NMDS plot, showed a strong selection of microbial communities by sample site (Figure 3-2a). Statistical analyses, using PERMANOVA analysis, supported this interpretation. The primary parameter affecting community diversity was site, which accounted for 30 % of the variation ($p < 0.001$).

Temperature appeared to be secondary, accounting for 14 % of variation ($p < 0.001$), and nutrient availability explained 5 % of the variation ($p<0.001$). The three sites formed three distinct clusters, each with sub-clusters separated by temperature and nutrient availability (Figure 3-2a).

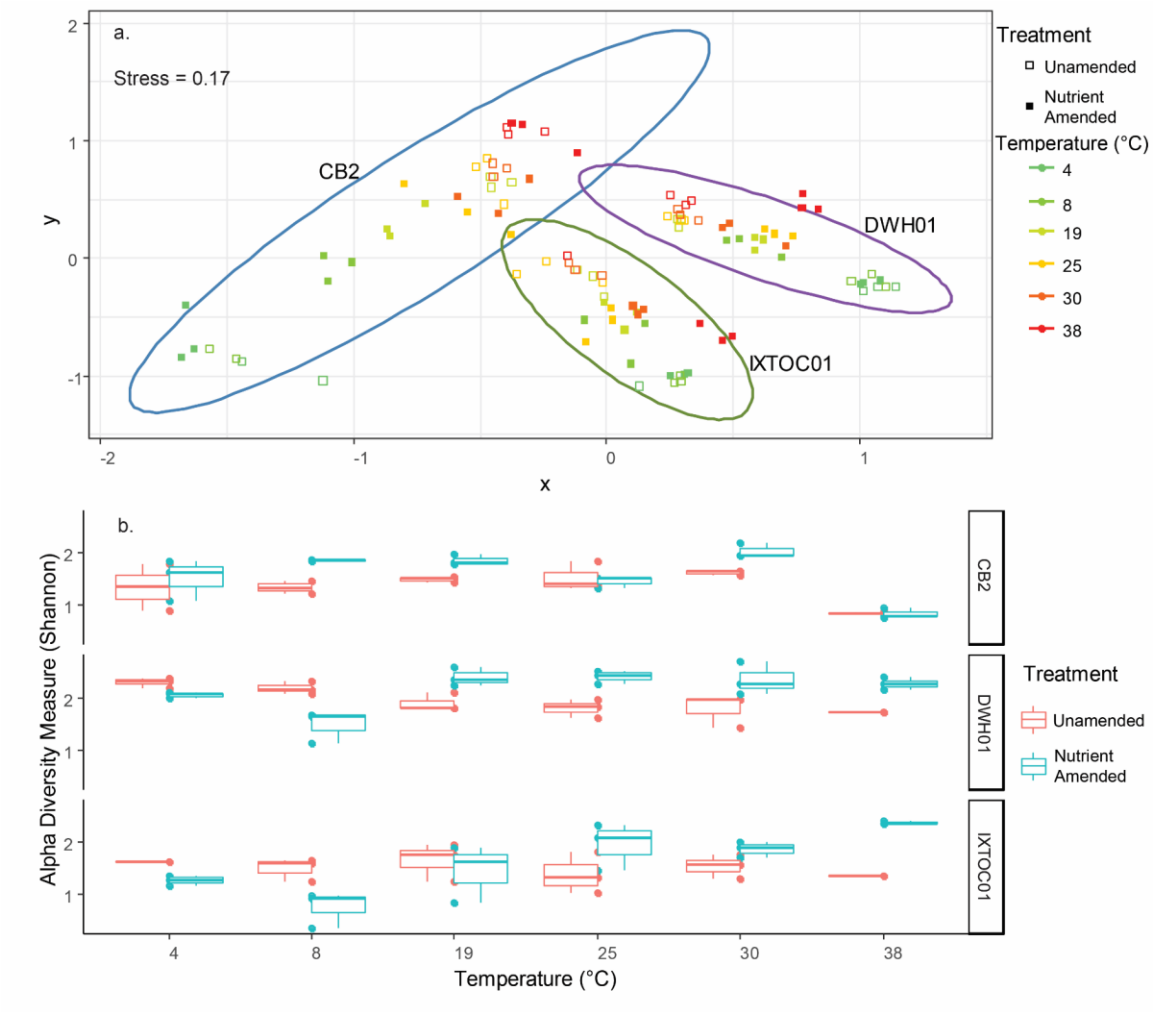


Figure 3-2 a) Comparison of microbial community composition between treatments in seawater microcosms. Similarities between microbial communities are displayed as the Bray-Curtis distance metric on a NMDS plot and b) Shannon entropy of microbial community alpha diversity. Boxplots show average values of triplicate samples. Error bars are standard deviations.

The taxonomic (alpha) diversity of microbial communities in each microcosm was generally elevated in nutrient amended treatments within the in situ temperature range of each site, while diversity remained the same or was lower at temperatures deviating from in situ conditions (Figure 3-2b). For example at lower temperatures of 4 and 8 °C, alpha diversity was elevated in the NA compared to the UN treatments at CB2, while diversity declined in NA compared to UN treatments at the GOM sites. Conversely, in CB2 incubations at 38 °C, in which respiration rates were lower than 30 °C, alpha diversity was not impacted by nutrient amendment, whereas alpha diversity was elevated in NA compared to UN treatments in the GOM seawater incubations.

At the phylum to class level, the *Gammaproteobacteria* dominated the microbial communities in all microcosms, ranging from 57 % to 98 % in relative abundance. Members of the *Alphaproteobacteria* and *Betaproteobacteria* were also important, contributing up to 22 % and 38 % of the total sequences retrieved, respectively (Figure 3).

Equal to higher alpha diversities were observed in nutrient amended CB2 microcosms comparing to unamended ones across the temperature range, and the Shannon Entropy plunged in both treatments at 38 °C (Figure 3-2b). At low temperatures (4 °C and 8 °C) in the CB2 microcosms, the genus *Colwellia* (denovo6) dominated both treatments, contributing up to 84 % of the total sequences retrieved. At mesophilic temperatures, *Thalassolituus* (denovo5) (46 % abundance at 25 °C), *Sulfitobacter* (denovo2) (33 % abundance at 25 °C), *Alteromonas* (denovo8) (26 % abundance at 19 °C), and *Acinetobacter* (denovo0) (19 % at 30 °C) were relatively evenly distributed in the NA treatment. The genus *Acinetobacter* constituted the majority of retrieved sequences in the

UN treatment at all mesophilic temperatures other than 38 °C (up to 59 % at 25 °C). The community diversity and evenness plunged in CB2 microcosms at 38 °C in both treatments, in parallel with lowered respiration rates, and the genus *Acinetobacter* (denovo0) predominated (88 % and 70 % relative abundance in UN and NA treatments, respectively).

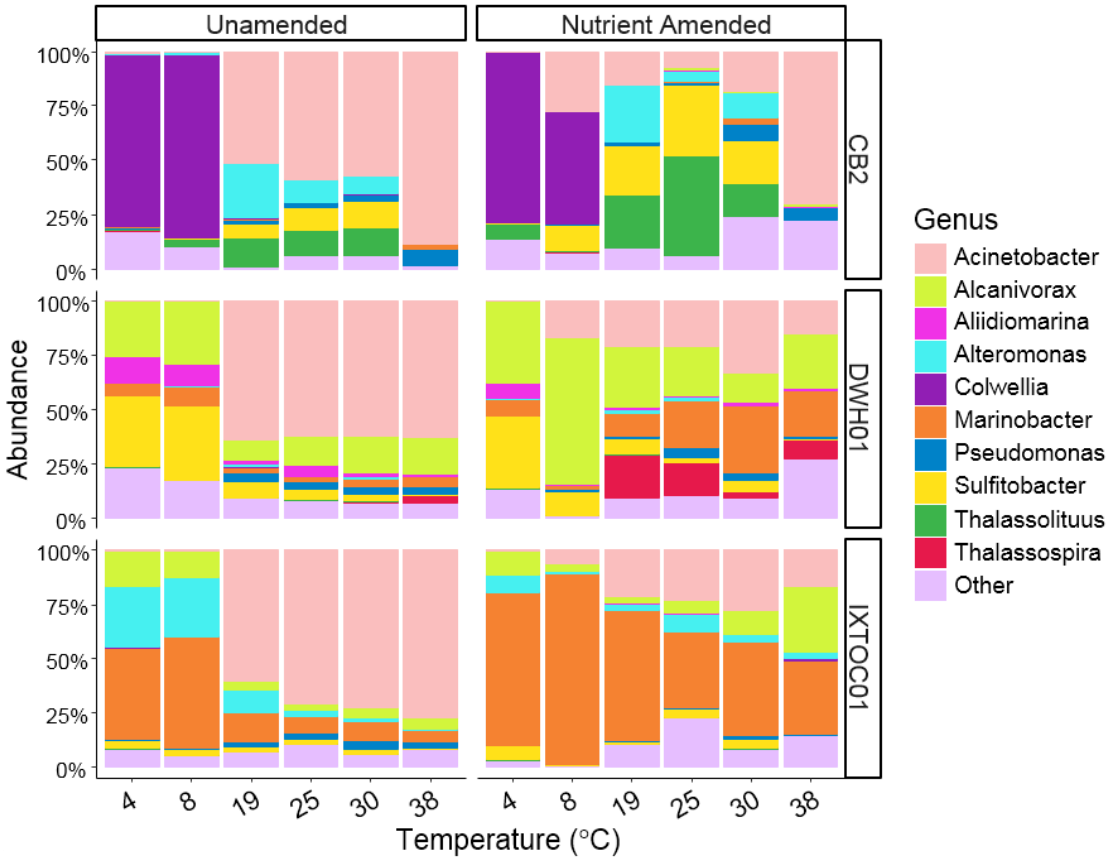


Figure 3-3. The relative abundance of genera in microcosms from (upper) CB2, (middle) DWH01, and (lower) IXTOC01 with treatments. Barplots show mean value of triplicate samples. Taxa are grouped at the genus level and relative abundance is calculated relative to total sequences.

Both GOM sites demonstrated similar patterns in alpha diversity (Figure 3-2b). In the DWH01 microcosms, the communities were very similar between treatments with a

dominance of two *Alcanivorax* OTUs (denovo4) (26 % and 38 % in UN and NA, respectively) and a *Sulfitobacter* OTU (denovo2) (33 % in both treatments) observed at 4 °C. At 8 °C, *Alcanivorax* was still a dominant contributor to the community, but different OTUs were detected in abundance, denovo3 and denovo4 in UN treatment (29 % relative abundance) and denovo4 in NA treatment (68 % relative abundance)). At mesophilic temperatures, the majority of UN treatments were again predominated by the genus *Acinetobacter* (denovo0) (up to 64 % relative abundance), followed by *Alcanivorax* (denovo3 only) (up to 17 % abundance). The relative importance of *Alcanivorax* (mostly denovo3) (up to 28 % abundance) and *Marinobacter* (denovo9) (up to 31 % abundance) increased in the NA samples.

In the IXTOC01 microcosms, a different group of the genus *Marinobacter* (denovo1) was dominant in the microbial communities at cold conditions, contributing up to 88 % and 48 % of total sequences in the NA and UN treatments, respectively. Another OTU within the genus *Alteromonas* (denovo10), which differed from abundant OTUs in CB2 microcosms, made a substantial contribution (up to 28 % abundance) to communities in the UN microcosms. Similar to the DWH01 microcosms, the UN samples were dominated by a single OTU of *Acinetobacter* at higher temperatures, contributing up to 68 % relative abundance of the community. In NA microcosms, an OTU (denovo1) affiliated with the genus *Marinobacter* was upregulated and contributed up to 58 % of total sequences.

3.5 Discussion

The fate and transport of discharged oil in seawater is determined by a complex interplay among hydrocarbon chemistry, the microbial food web, and ambient

oceanographic parameters [143]. The complex interactions between environmental factors that regulate the efficiency of microbially-mediated hydrocarbon degradation are not understood[36]. Lack of knowledge regarding the controls of biodegradation, and spatiotemporal variation among these controls, is a critical obstacle to effective parameterization of predictive models that guide oil spill response efforts. Therefore, in this study, petroleum hydrocarbon (HC) biodegradation potential was investigated at three study sites, chosen based on hydrocarbon exposure history, potential hydrocarbon spill risk, and climatic region. DWH01 and IXTOC01 represent subtropical and tropical sites, respectively, where the largest accidental marine oil spills in history occurred in the Gulf of Mexico (GOM) [4,148]. The Beaufort Sea represents a pristine, permanently cold polar site, being considered for off-shore drilling platforms on the continental slope [149]. Site CB2 was chosen as it shared many oceanographic similarities with DWH01. Like DWH01, CB2 is a pelagic site located on the continental slope and remote from shore. In addition, the region surrounding CB2 is socio-economically and ecologically relevant as a highly productive marine ecosystem in the path of Northwest Passage [1]. Concerns are being raised regarding the elevated risk of hydrocarbon contamination due to increasing oil exploration and marine transportation activities in the Arctic [1,150]. A systematic understanding of the rates and controls of hydrocarbon biodegradation and microbial community response will be critical for assessing environmental risks in new areas of oil exploration.

3.5.1 Environmental controls of biodegradation

The Deepwater Horizon (DWH) oil spill catalyzed a resurgence in studies of the rates and controls of hydrocarbon degradation [143,151]. Since the Gulf of Mexico is exposed to a substantial amount of natural oil seepage, it was suggested immediately after the DWH

disaster that microbial communities are "primed" or adapted for an intrinsically high potential for oil biodegradation [8]. Based on analysis of SSU rRNA gene amplicons, numerous studies have shown that hydrocarbon-degrading bacteria are likely to be ubiquitous albeit rare in Gulf ecosystems, including areas that are not immediately exposed to natural seepage [143,152]. Indigenous microbial communities in the immediate vicinity of natural hydrocarbon seeps are physiologically adapted to the processing of HCs [152]. However, natural hydrocarbon seepage occurs in all of the world's ocean basins. Moreover, phytoplankton represent a widespread source of hydrocarbons such as alkanes throughout the world's oceans [132,141], and thus hydrocarbon-degrading bacteria are also likely to be equally distributed. Evidence is lacking to support the hypothesis that Gulf microbial communities are inordinately "primed" for hydrocarbon degradation in comparison to other ocean basins.

In this study, the activity and growth of microorganisms in oil-amended microcosms of pristine Arctic seawater nearly always exceeded that of seawater sampled from the northern and southern Gulf at sites of major oil spills. These data should be verified by direct measurements of petroleum hydrocarbons and clearly further sampling across ocean basins is needed to constrain spatiotemporal variation in hydrocarbon degradation potential. Nonetheless, our results do not support the paradigm that the Gulf is "primed" for degradation in comparison to other ocean basins. In contrast, this study demonstrates a strong selection for hydrocarbon-degrading microbial communities by site, dependent upon factors other than the history of exposure to high levels of hydrocarbons. Beta diversity analysis showed that microbial communities were most strongly separated by the site of origin from the polar to tropical sites. Alpha diversity in the microcosms responded

to the in situ temperature range present at each site, suggesting adaptation to the ambient temperature at each site. Finally, the composition of hydrocarbon-degrading communities was clearly distinct between sites, especially when considering the ambient temperature range of each site. For example, *Colwellia* only dominated in CB-2 microcosms incubated at temperatures (4, 8 °C) close to those of the permanently cold Arctic, whereas other well-known hydrocarbon-degraders of the *Gammaproteobacteria* (*Acinetobacter*, *Alcanivorax*, *Marinobacter*, *Alteromonas*) dominated at mesophilic temperatures common to the subtropical to tropical GOM. Hydrocarbon-degrading bacteria are ubiquitous in the world's ocean as well as in terrestrial environments [153]. Since organic matter produced by extant photosynthetic organisms contains compounds analogous to HCs such as alkanes [132], it is likely that microbial communities in any ecosystem exposed to such compounds are "primed" or adapted to hydrocarbon degradation to some extent. Thus, the potential for oil biodegradation is more likely limited by the complex interplay of environmental parameters unique to each ecosystem.

We suggest that the site-specific response can be explained by the ecological strategy employed by indigenous microbial communities [154]. We posit that microbial communities in the Arctic adopt an r-strategy, which allows for rapid responses over an abbreviated seasonal cycle; whereas communities in the GOM are K strategists adapted to a more stable environment. During the spring bloom in the Arctic, phytoplankton carbon is injected into surface waters in a short period [155–157]. These observations led to the hypothesis that microorganisms were adapted to a short period of intensive carbon input (i.e. spring bloom), and therefore could immediately respond to substrate input. On the contrary, both GOM sites were heavily influenced by organic matter inputs from rivers, leading to more

stable substrate availability [158]. Kleindienst et al. also suggested that microorganisms which employ an r-strategy respond faster than microorganisms using K-strategy during oil spills [152].

Another possibility for elevated hydrocarbon degradation potential could be related to nutrient availability. Our observation of slightly elevated nutrient concentrations at CB2 corroborated previous work on global nutrient limitation patterns [31]. Generally, nitrogen and phosphorus levels are depleted at the surface in low-latitude tropical oceans by extensive photosynthesis [31], while low temperatures and deficiencies in light lead to high nutrient levels in the polar region. This, in fact, provides necessary inorganic nutrients that are critical for hydrocarbon degradation [32].

Temperature has long been recognized as a critical parameter that regulates hydrocarbon biodegradation [11]. It influences biodegradation through effects on physical-chemical properties of oil as well as on microbial structure/ function [12]. Many studies, primarily conducted in the laboratory, indicate that temperature strongly regulates the capacity and efficiency of petroleum hydrocarbon degradation in seawater [20]. However, results are equivocal and kinetic constraints may not be as important as previously perceived [16,27,63–65]. For example, Techtmann et al. found very low rates of alkane and PAH degradation at 4 °C in deepsea water in comparison to rates measured at 25 °C [21]. Conversely, other studies observed equally rapid biodegradation rates, with half lives of one week for alkanes and one to two weeks for PAHs, under cold conditions (4 °C to 6 °C) in shallow waters from a Norwegian fjord in comparison to deep oil plumes from the DWH spill in the GOM [8,159]. Bagi et. al. (2014) found biodegradation rates of the PAH,

naphthalene, were three times higher in Arctic surface seawater in comparison to temperate Atlantic seawater across a range of colder temperatures (0.5 to 15 °C) [23].

In this study, distinct temperature responses were observed in surface seawater microcosms based on geographical origin, from the subtropical to tropical GOM sites (DWH01, IXTOC01) to the polar Beaufort Sea (CB2). We observed universally more rapid degradation rates in surface sweater from the Arctic in comparison to subtropical to tropical sites, in corroboration of Bagi et al. [23]. Results from CB2 microcosms indicated that the *in situ* microbial community from a permanently cold Arctic environment could maintain relatively rapid degradation rates across a large range in temperature, indicating cold adaptation [1,19,20,112,160,161]. In contrast, little degradation activity was observed at lower temperatures (4, 8 °C) in microcosms from the GOM sites, suggesting adaptation to mesophilic temperatures [162]. Site-specific temperature adaptation was confirmed by comparing the ratio of degradation rates between 4 °C and the optimum temperatures (R_{Topt} /R_{4C}) (Table 3-1). Lower ratios in CB2 microcosms pointed to less change in microbial activity across the range in temperatures, while higher values in both GOM sites indicated more variation with temperature [163]. These results are corroborated by the *in situ* temperatures of the sampling sites. Whereas the average of annual sea surface temperature (SST) is approximately 25 °C in the GOM [164], temperature remains permanently cold at < 1 °C at CB2 in the Beaufort Sea. The Q_{10}, the measurement of rate of change in reaction rates of 10 °C increase, values measured in the current study fell within the middle range of literature values [20]. Q_{10} is temperature-dependent and its value decreases when approaching growth limits [165]. This could explain the low Q_{10} for CB2, whose optimum temperature was lower than the other two sites. Neither temperature nor nutrient

availability changed the cell specific respiration, suggesting a similar degradation efficiency.

The consensus appears to be that it is not possible to interpret rates of oil biodegradation as first order with respect to temperature due to confounding factors such as nutrient limitation, the solubility of HCs, and microbial community composition in various studies [20]. Variations in methodology and experimental conditions also likely contribute uncertainty.

All rate constants from this study were lower than *in situ* estimates of biodegradation from the DWH oil slick [74]. This inconsistency could be explained in at least two ways. First, estimates from previous studies were based mainly on direct measurements of HCs while our study used respiration rates as a proxy. Only a portion of HCs will be mineralized through microbial respiration while the remainder may be assimilated into biomass, and therefore a lower rate was to be expected [166]. Also, hydrocarbon degradation is a multi-step process, which produces many intermediates and is often incomplete, thereby leading to a reduction in CO_2 production[104,167]. In fact, previous studies have shown that approximately 10 % of GC amendable oil was mineralized to CO_2 during a 60-day incubation [19]. Second, our microcosms were amended with higher oil concentrations than those measured in the DWH slick, which likely resulted in lower biodegradation rates due to nutrient limitation[13], and this is corroborated by the stimulation in rates from NA relative to UN treatments. Nutrient limitation was expected in the incubations, since high oil concentrations are likely to exhaust available nutrients [13]. Depletion in available nutrients might lead to decreased microbial productivity [31]. Many previous studies have reached the same conclusion [32,33,35,74]. Liu et al., however, found contradictory results, where PAH

degradation was less favored by higher nutrient levels [26]. Due to the high alkane content in the source oil, however, the overall degradation rate could still be enhanced by nutrient amendment.

3.5.2 Microbial community dynamics across site, temperature, and nutrient availability

The dominance of the class *Gammaproteobacteria* was expected, as most well-known marine hydrocarbon-degrading bacteria reside in this group [1,8,58,159]. Previous work indicates that members of the *Gammaproteobacteria* are well adapted to changing environmental conditions and respond rapidly to pulses in organic matter input [168,169]. At higher taxonomic resolution, shifts in community composition reflected adaptation in response to environmental conditions. At cold temperatures closest to ambient conditions in polar surface waters, the genus *Colwellia* was selected in all microcosms. The genus *Colwellia* contains many known psychrophilic members [170,171], and was often found in oil contaminated seawater at cold conditions [8,23,81,105]. At higher temperatures in incubations of Arctic waters, *Colwellia* was outcompeted, and other genera (*Thalassolituus* and *Sulfitobacter*) dominated the communities with nutrient addition. The genus *Thalassolituus* is a obligate hydrocarbon-degrading group [172], often associated with oil contaminated sites at both 4 °C and 20 °C [8,33]. On the contrary, although often found in oil contaminated environments [108,173,174], the genus *Sulfitobacter* has not yet been shown to degrade HCs. At all temperatures, the relatively large increase in bacterial abundance indicated the microbial community in polar waters could rapidly respond to hydrocarbon input. Yager et al. suggested that multiple thermal groups, psychrophilic and psychrotolerant microorganisms, co-exist in the Arctic sea and compete with each other for substrates [175].

Our observations of the response of hydrocarbon-degraders across a range in temperatures are in agreement with this conclusion.

Unlike the Arctic microcosms, no distinct microbial group at the genus level was selected by low temperature in incubations of GOM seawater. The enrichment of known hydrocarbon-degraders, including *Alcanivorax, Alteromonas, Marinobacter,* and *Thalassospria*, indicated that these groups are adapted to high oil concentrations and elevated nutrient concentrations. These findings are corroborated by previous studies of planktonic ecosystems in the Gulf, where these genera were enriched under heavily oiled conditions either *in situ* [28,176] or *ex situ* [122].

At mesophilic temperatures regardless of the site sampled, all unamended microcosms were dominated by the genus *Acinetobacter*, and the group persisted in all nutrient-amended microcosms. *Acinetobacter* is well known to degrade a range of HCs including long-chain alkanes [177] and PAHs, such as phenanthrene and pyrene [178]. This genus was characterized as a group of non-motile bacteria [179], capable of emulsifying as well as adhering to hydrocarbon substrates [180]. The high relative abundance of *Acinetobacter* in unamended treatments suggests that it outcompetes other hydrocarbon-degraders in heavily oiled and nutrient-depleted environments. In agreement with this finding, *Acinetobacter* was observed in high abundance in oil slicks and heavily oiled beach and saltmarsh sediments [58,181,182], but not in the dispersed oil plumes resulting from the DWH oil spill in May, 2010 [28]. This corroboration suggested that our experiment was environmentally relevant and should be considered for assessing environmental risks.

Previous work hypothesized that hydrocarbon-degradation activity is a strain-specific trait [15,58,139,152]. Our results supported this hypothesis. Distinct OTUs from several genera were selected for in our microcosms according to site, temperature, and nutrient availability. For example, one OTU of *Sulfitobacter*, denovo2, was abundant in CB2 and DWH01 microcosms, while the another group, denovo11, was only present in the IXTOC01 incubations at low abundance. *Marinobacter* OTUs also showed a strong site effect. Although this group was important in microcosms of both GOM sites, denovo9 was only present in DWH01 while denovo1 dominated IXTOC01. Temperature and nutrient conditions also selected for specific OTUs. At DWH01, *Alcanivorax* denovo4 was relatively important in NA treatments at cold temperatures, while denovo3 was more abundant in UN treatments. The relative enrichment of diverse OTUs might be a result of selection of specialized sub-populations, which adopt a different ecological strategy for each environmental niche [152].

3.6 Conclusions

Here we demonstrate that the microbial community response to oil contamination in surface waters of major oil exploration regions is site specific and dependent upon ambient conditions, temperature and nutrients. The activity, diversity, composition, and abundance of microbial communities were all strongly selected by site in seawater microcosms, suggesting adaptation to the local environment. The results indicate that the site of origin may override nutrient availability and temperature in dictating hydrocarbon degradation potential. Surprisingly, the highest hydrocarbon degradation rates were observed in pristine polar waters. Thus, our results call into question the role of chronic oil pollution in the "priming" Gulf of Mexico waters for oil biodegradation. Known hydrocarbon-degraders

of the *Gammaproteobacteria* dominated the microbial communities of all microcosms. While the putative psychrophilic group *Colwellia* dominated under cold conditions in polar seawater regardless of nutrient content, other groups (*Acinetobacter*, *Alcanivorax*, *Marinobacter*, *Alteromonas*) were important at mesophilic temperatures and communities responded at all sites to nutrient amendment. The genus *Acinetobacter* was observed in high abundance in the current study and field observation, but not found at low oil concentrations, suggesting different groups of microorganisms might be responsible for oil degradation under elevated oil concentration. It is important to note that this study represents only a snapshot of the likely spatiotemporal variation of the three sites, and should not be extrapolated to the entire geographical region. Future studies should incorporate direct measurements of HCs and focus on further verification of the complex interplay between environmental controls of biodegradation.

CHAPTER 4. IN SITU PRESSURE ACTS AS A SELECTIVE FORCE ON THE STRUCTURE AND FUNCTION OF DEEPSEA MICROBIAL COMMUNITIES THAT MEDIATE PETROLEUM HYDROCARBON DEGRADATION

Xiaoxu Sun, Sheng Dai, and Joel E. Kostka

4.1 Abstract

The Deepwater Horizon (DWH) disaster represents the largest accidental marine oil spill in history. One of the unique characteristics of the DWH spill is the depth at which it occurred, approximately one mile below the sea surface. At the wellhead, overlying water (~1,500 m) leads to elevated hydrostatic pressure, equivalent to 150 times that of atmospheric pressure. Our understanding of the impacts of hydrostatic pressure on the structure and function of microbial communities remains in its infancy. The goal of this study is to investigate how *in situ* microbial communities respond to oil contamination at pressures observed in the deepsea in comparison to atmospheric pressure. Shallow and deepsea sediment, as well as deepsea water, were collected in the northern and southern Gulf of Mexico. Seawater and/or sediments were incubated in specially designed high-pressure incubation chambers, with or without oil addition, at atmospheric (0.1 MPa) or elevated (10 MPa) pressure, equivalent to the pressure expected at 1,000 m water depth. Aerobic respiration, determined as oxygen consumption, was used as a proxy for hydrocarbon biodegradation. Results indicated that samples from the deepsea showed a distinct response to pressure in comparison to shallow origin samples. While respiration in

shallow sediment showed little impact of elevated pressure, activities measured in deep origin samples were enhanced by 20 % to 80 % at the *in situ* pressure of 10 MPa. The composition of metabolically-active microbial communities, as determined by next generation sequencing of rRNA extracts, responded to oil addition and elevated pressure, suggesting these parameters preferentially select for specific microbial populations.

Known hydrocarbon-degrading bacteria within the class *Gammaproteobacteria* (*Colwellia*, *Cobetia*, uncultured *Oceanospirillaceae*) were abundant and active in treatments. *Cycloclasticus*, C1-B045 of the family *Alteromonadaceae*, *Oleispira*, and an uncultured population of the *Oceanospirillaceae* showed high relative abundance in oil-amended incubations, while *Psychromonas*, *Colwellia*, and uncultured members of the *Oceanospirillaceae* responded to pressure treatment. *Oceanospirillaceae* OTUs retrieved from bottom water incubations showed high sequence identity to taxa that were detected in the deep oil plumes from the DWH disaster. This study is among the first to use *ex situ* incubations to determine the impacts of high pressure on the structure and function of indigenous microbial communities, which should aid in refining model predictions of oil fate to take into account the effects of pressure.

4.2 Introduction

The average depth of the world's oceans is approximately 3,800 m, and the deepsea represents the largest habitat for biota on earth [37]. As evidenced by the renowned "lunch box" experiment, where human food was preserved in a submarine that fell to the seafloor for an extended period, pressure has long been implicated as an important factor for the regulation of microbial activity [12,38]. The first study of the impacts of pressure on marine microorganisms was reported by Zobell and Johnson in 1949 [39]. The effects of pressure

were determined for bacteria isolated from terrestrial and marine habitats, and strains from the deepsea shown to grow under extremely high pressure. Microorganisms that thrive under elevated pressure conditions were first termed "barophilic", and this was later revised to piezophilic by Yayanos [40].

In the deepsea, surface-derived piezosensitive or piezotolerant bacteria are believed to coexist with native piezophilic bacteria [38]. Grossart et al. observed that a gradual increase in pressure selected different microbial populations in *ex situ* incubations[41]. Surface-derived populations can be transported through the water column to the deepsea via natural organic aggregates [42]. In an incubation testing the pressure effect on the surface seawater, exposing surface-derived enzymes to elevated pressure was shown to inhibit enzyme activity at pressures above 1 MPa, and activity was reduced by 90 % at 10 MPa [43]. Similar results were obtained in many other studies [44,45]. In contrast, deepsea bacteria adapt to elevated pressure by modifying either lipid [46,47] and/or protein structures [48,49]. Adaptation to ambient pressure was implicated by the surprisingly rapid substrate incorporation and respiration observed in the deepsea [50]. Therefore, in order to achieve accurate measurements on deepsea microbial activity, *ex situ* incubations should be conducted at *in situ* condition [44]. By far, only a small portion of studies, however, performed experiments at elevated pressure, possibly due to the difficulty to obtain deepsea samples and the requirement of special pressure cells to maintain high pressure.

The Deepwater Horizon oil spill (DWH) accident represents the largest accidental marine oil spill in the US history [71,82]. With the unprecedented 3.19 million barrel equivalents of oil released into the Gulf of Mexico (GOM), various environments were exposed to hydrocarbon contamination[6]. One of the unique aspect of the DWH was the

depth where it happened [4]. The spilled oil was injected into the GOM at the seafloor, where is 1.5 km below sea surface. According to the literature, approximately 10 % of the total released oil rose to the surface and formed slicks [7], 35 % formed dispersed oil plumes in the deepsea [8,9], and up to 47 % was trapped in sediments at the seafloor [10]. At this depth, the overlaying seawater creates tremendous pressure, which equals 150 times of the atmospheric pressure. Despite the fact that literatures stated the *ex situ* experiments should be conducted at ambient condition, there are no experiment has been conducted at the *in situ* pressure [108]. In general, our current knowledge of hydrocarbon degradation at elevated pressure is still sparse. By far, most of the high pressure hydrocarbon degradation experiment was limited to pure cultures, mostly surface-derived, and shallow seawater [46,85,183–187]. Studies on hydrocarbon degraders from the deep-sea with culture-independent method is rare. Bowles et al. found elevated methane degradation at elevated pressure, but this most likely cause by higher methane solubility at 5 MPa [188]. Therefore, the question on how pressure affected oil diagenesis during DWH was clear.

The objective of this study is to provide useful insights and measurement on the hydrocarbon degradation potentials affected by at ambient pressure comparing to the atmospheric pressure. Sediment and seawater samples were incubated with or without oil addition and incubated at 0.1 MPa and 10 MPa. Both geochemical and biological evidences were integrated together to obtain insights of the degradation process at ambient conditions.

4.3 Methods

4.3.1 Sample collection

Sediment and water samples were collected during multiple cruises (WB0816 and WB0817) on the R/V Weatherbird II in 2016 and 2017. Deepsea samples were collected near the Deepwater Horizon (DWH) wellhead and in the DeSoto Canyon area, which lies to the northeast of the wellhead in the northeastern GOM. The water depth of the sample sites was 1,100 m at PCB06 (N 29° 11', W 87° 26'), and 1,500 m at DSH10 (N 28° 58', W 87° 52'). The ambient temperature was 4 °C. Shallow sediment was collected in southern GOM on research cruise R/V Weatherbird II in 2016. The sampling site, LT4 (N 19° 21', W 92° 16'), was located in the Bay of Campeche, Mexico. The LT4 samples were collected at 30 water depth, where the ambient temperature was 30 °C.

Water samples were collected in Niskin bottles attached to a CTD sampling rosette (Sea–Bird Electronics INC., Washington, USA) at 10 m above seafloor. Sediment cores were collected using a multicoring device. During each deployment, a maximum of eight sediment cores were collected within 1 m diameter area of the seafloor. The retrieved cores were immediately sectioned into three depth intervals, 0 – 3 cm, 3 – 5 cm, and 5 – 10 cm depth, with an extruder. Sediment sections were stored in sterilized plastic containers or Ziploc bags. All samples were immediately stored at 4 °C until use.

4.3.2 Experimental approach

The experimental approach generally followed previously described methods, with modifications [189]. Briefly, sediment samples were mixed with filtered bottom waters of the same site in a ratio of 1:3. Five ml of slurry/water were then added to heat-sealable polypropylene ultracentrifuge tubes (Beckman-Coulter, California, USA) and separated into four treatments: high pressure amended with 2 µl of oil (HPO), high pressure without

oil (HPNO), atmospheric pressure amended with 2 µl of oil (APO), and atmospheric pressure without oil (APNO). The oil used in the experiments was Macondo MC252 surrogate oil [86]. To pressurize the samples, sealed tubes were placed into high pressure chambers. The chambers were then filled with sterile, aerated Nanopure water and compressed to 10 MPa, which is equivalent to 1000 m water depth (HP), using a high-pressure syringe pump (Teledyne ISCO 500D). Parallel chambers were prepared for incubations at atmospheric pressure (AP) in parallel with the HP treatments. Before incubation, all chambers were pre-incubated overnight at ambient temperature (4 °C for deepsea samples and 25 °C for shallow samples). Killed controls were prepared by autoclaving the sample on three consecutive days before incubation.

Aerobic respiration as determined by oxygen consumption was employed as a proxy for hydrocarbon degradation [8,19]. Triplicate samples were sacrificed at each time point and oxygen concentrations were measured using a Presens oxygen transmitter with needle type sensor (Presens, Germany). Total oxygen consumption was determined from oxygen consumption in each incubation tube as well as diffusive oxygen loss in the water surrounding the tubes in each pressure cell. Diffusive oxygen loss was determined by the difference in oxygen concentration in the water surrounding the tubes before and after incubation, with the assumption of equal diffusion into each tube. Degradation rates were calculated from the linear regression of oxygen concentration with time.

4.3.3 Nucleic acid extraction and next generation sequencing of SSU rRNA amplicons

At termination of each incubation, the volume from each tube was pelleted by centrifugation and nucleic acids were extracted with the ZymoBIOMICS DNA/RNA

Miniprep Kit (Zymo Research, California, USA), following the manufacturer's protocol. Both DNA and RNA were extracted. The extracts were stored at -80 °C prior to downstream applications. The RNA was reverse transcribed to complimentary DNA (cDNA) with qScript XLT cDNA supermix (Quantabio, MA, USA). Extracted DNA or cDNA was quantified with the Qubit HS assay kit (Invitrogen, Carlsbad, CA, USA) and 10 ng per reaction was used to generate SSU rRNA amplicons. Prokaryotic community composition was determined by applying a high-throughput sequencing-based protocol that targets PCR-generated amplicons from the V4 variable regions of the SSU rRNA gene using the primer set CS1_515F (5'-ACACTGACGACATGGTTCTACA_GTGCCAGCMGCCGCGGTAA) and CS2_806R (5'-TACGGTAGCAGAGACTTGGTCT_GGACTACHVGGGTWTCTAAT) [94,95]. PCR conditions were as follows: 5 minutes of denaturation at 95 °C, 28 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 45 seconds, elongation at 72 °C for 30 seconds, and followed by 7 minutes of final elongation at 72 °C. The resulting SSU rRNA gene amplicons were barcoded with unique 10-base barcodes (Fluidigm Corporation, CA, USA), pooled into equal DNA aliquots, and sequenced on an Illumina MiSeq2000 platform at the DNA services facility of the University of Chicago according to established methods [96–98].

4.3.4 Sequence and statistical analysis

Sequences were analyzed and statistical comparisons were conducted using a set of R packages, including dada2, Phyloseq, DESeq2, ggplot2, and vegan [146,190]. Briefly, the raw sequences were trimmed and filtered to remove primers and low quality regions. The

filtered reads were demultiplexed. Then the forward and reverse reads were merged and chimeras were removed. The sequences were assigned taxonomy using the SILVA database, aligned in the package DECIPHER, and the alignment was exported to qiime for building of phylogenetic trees [94]. The community data was fitted onto an ordination for identification of the corresponding environmental variable in vegan [191]. PERMANOVA analysis was used for statistical comparison of microbial community dissimilarity [118]. Then the beta dispersion of the samples was calculated. Predominant microbial populations that varied with oil and pressure treatment were identified by a similarity percentage (SIMPER) test, and the selected sequences were analyzed by a Kruskal-Wallis test to detect significant variations by treatment. Additional strains that were significantly affected by treatment were identified using DESeq2 package [192]. The Kruskal-Wallis test determines the mean and standard deviation of relative sequence abundance for predominant microbial populations, while DESeq2 measures log2 differences of the abundance. Therefore, the two tests may provide complementary results.

4.4 Results

4.4.1 Hydrocarbon degradation measured in oxygen consumption

Biodegradation rates tracked with the predicted organic matter content of each seawater or slurry incubation. Sediment incubations demonstrated much higher overall biodegradation rates (calculated as the difference between oil treatments and no oil controls) in comparison to seawater incubations (Figure 4-1). The most rapid biodegradation rates were observed in incubations of shallow sediments (LT4) at room

temperature (25 °C). The impacts of natural organic matter could be observed while comparing the oil amended treatments to those without oil. Whereas respiration rates were similar between all treatments in shallow sediments, rates were 2 to 6 times higher in oil-amended treatments in comparison to the no oil in incubations of bottom water or deepsea sediments. Respiration rates in LT4 incubations averaged 435.2 ± 28.1 µmol O_2/L sediment/day, 452.3 ± 33.9 µmol O_2/L sediment/day, 377.6 ± 25.792 µmol µmol O_2/L sediment/day and 460.1 ± 36.44 µmol O_2/L sediment/day for HPO, HPNO, APO, and APNO, respectively. The differences observed between the treatments were statistically insignificant.

Biodegradation rates were slower in incubations of deepsea sediments in comparison to shallow LT4 sediments, probably due to the lower incubation temperature combined with a lower sedimentary organic matter content. Respiration rates in PCB06 incubations averaged 260.4 ± 12.1 µmol O_2/L sediment/day, 122.7 ± 6.3 µmol O_2/L sediment/day, 197.8 ± 13.5 µmol µmol O_2/L sediment/day and 89.2 ± 4.5 µmol O_2/L sediment/day for HPO, HPNO, APO, and APNO treatments, respectively. In DSH10 incubations, respiration rates of 367.0 ± 36.1 µmol O_2/L sediment/day, 143.0 ± 5.6 µmol O_2/L sediment/day, 299.0 ± 20.0 µmol µmol O_2/L sediment/day and 127.4 ± 5.2 µmol O_2/L sediment/day were observed for HPO, HPNO, APO, and APNO treatments, respectively. For all incubations of deepsea sediments, the difference between each pair of treatments (HP vs. AP and Oil vs No Oil) was significant ($p < 0.05$) according to the Tukey HSD comparison.

Respiration rates in incubations of DSH10 bottom water were much slower in comparison to sediment incubations. No significant differences were observed in the no oil

treatments between pressures (6.9 ± 0.5 µmol O_2/L sediment/day at HPNO and 6.1 ± 0.5 µmol O_2/L sediment/day of APNO). However, differences in oil-amended treatments between the two pressures were significant ($p < 0.01$). In DSH10 BW, the absolute biodegradation rate increased by 1.8 times at HP (33.3 ± 0.85 µmol O_2/L sediment/day) compared to AP (20.4 ± 1.6 µmol O_2/L sediment/day).

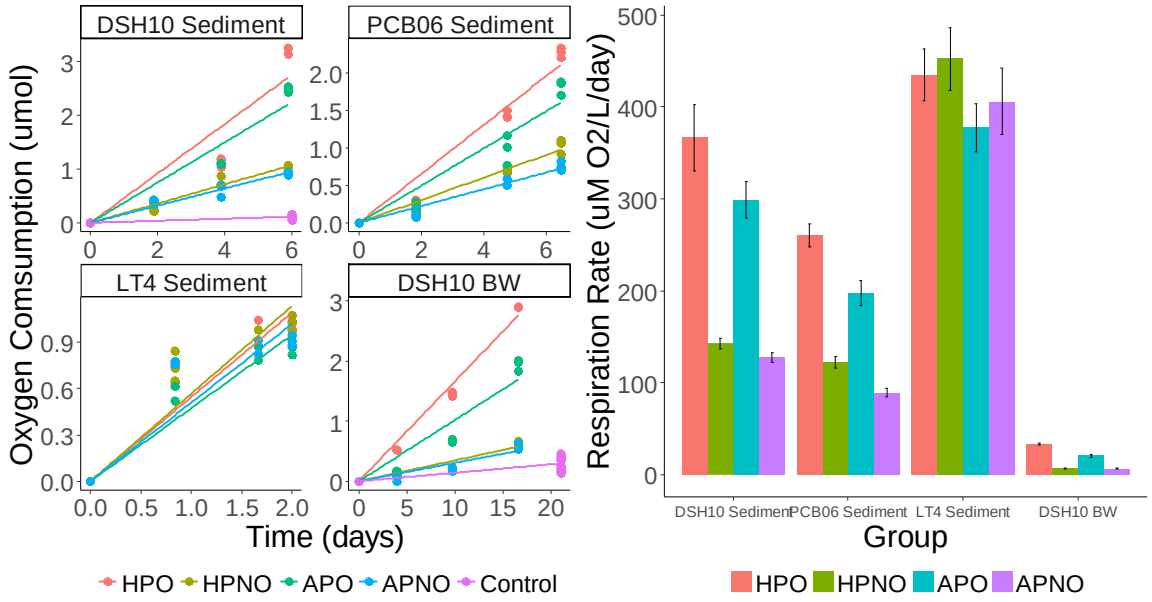


Figure 4-1. Biodegradation as determined by oxygen consumption in incubations of sediment and seawater. The left panel shows oxygen consumption with incubation time. Solid lines represent the fitted linear regression. The barplots in the right panel present the average respiration rates for each incubation calculated from the slope of a linear regression. Error bars are standard deviations of the fitted curve.

4.4.2 Microbial community composition

Taxonomic (alpha) diversity at the RNA level generally paralleled the corresponding diversity at the DNA level in all treatments, according to observed species or the Shannon index (Figure 4-2). Diversity was generally lower in the oil-amended treatments in comparison to the no oil treatments in DSH10 and PCB06 incubations.

Conversely in LT4 incubations, taxonomic diversity was always equal to or higher in oil-amended treatments in comparison to the no oil. In addition, while diversity was always lower at the RNA level in comparison to the DNA level in DSH10 and PCB06 incubations, the Shannon Entropy was higher at the RNA level for site LT4 in all treatments, with the exception of APNO, whereas observed species diversity was equal to or lower at the RNA level in comparison to DNA in LT4 incubations. (Figure 4-2). For sediment incubations of all sites, no clear pressure nor oil amendment effect was observed on microbial community composition, as evidenced by PCoA plots of Bray-Curtis distance metrics (Figure 4-3). In incubations of DSH10 bottom water, however, a clear separation of microbial communities was visualized between different treatments. While oil addition represented a major selective force ($p < 0.05$ for PERMANOVA test), pressure treatment also showed a substantial impact on microbial community composition ($p = 0.08$ in PERMANOVA test) (Figure 4-4).

For DSH10 sediment community at the RNA level, PERMANOVA analysis suggested that both oil addition and pressure treatment significantly affected community composition. Beta dispersion indicates that differences were mainly contributed by distance between groups for oil and no oil samples, whereas differences between HP and AP treatments were likely due to dispersions within the groups. Two microbial groups made a significant contribution to the differences, showing enrichment in the oil-amended treatments. Sequences affiliated with the genus *Cycloclasticus* of the family *Piscirickettsiaceae* (6 % and 7 % for HPO and APO, respectively and not found in no oil treatments), showed high sequence identity to *C. spirillensus* (99 %), while sequences retrieved from the C1-B045 clade of the family *Alteromonadaceae* were most closely

related to a cultured representative of *Marinobacter zhanjiangensis* (94 %). OTUs from C1-B045 comprised approximately 2.5 % relative abundance in both HPO and APO RNA samples, but were not detected in the no oil treatments. The dominant OTUs in the community were affiliated with the genus *Colwellia*, which contributed approximately 71 % of sequences retrieved from all treatments. The most abundant *Colwellia* OTU was closely related to *C. psychrerythraea* (99 %), which declined in abundance at elevated pressure (Supplementary Figure 4-1).

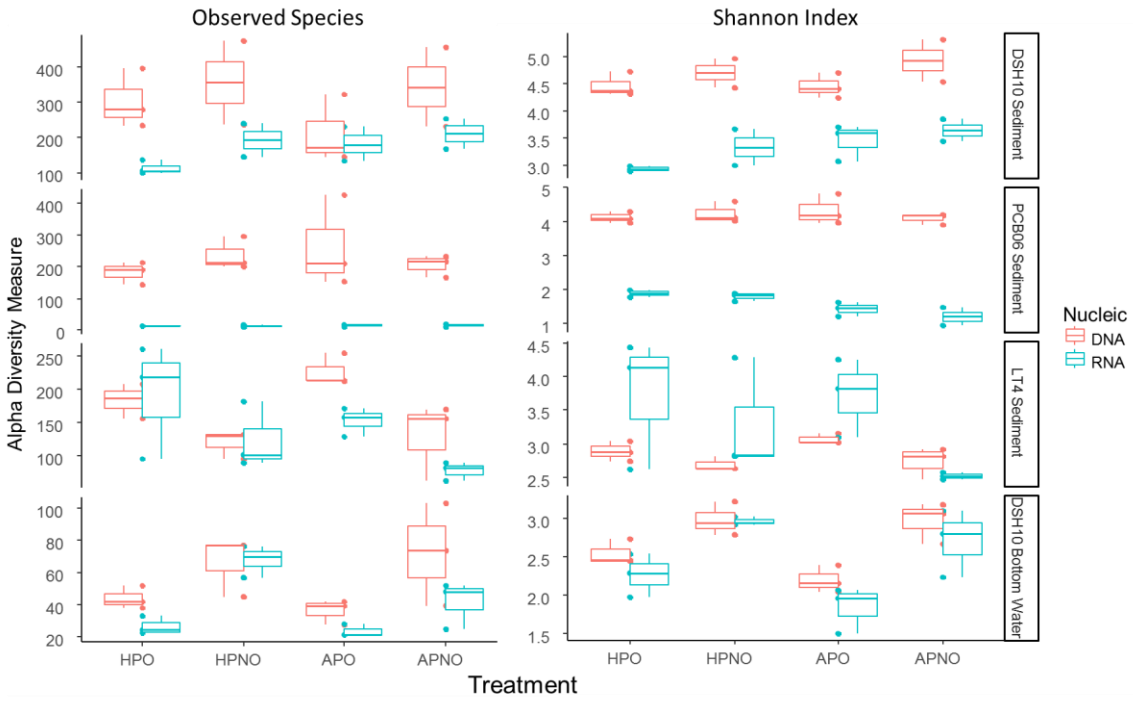


Figure 4-2. Taxonomic (alpha) diversity of microbial communities from incubation treatments expressed as observed species (left panel) and Shannon indices (right panel). Boxplots represent the average values of triplicate samples. Error bars are standard deviations.

In incubations of PCB06 sediments, larger differences in community composition were observed at the RNA level, and impacts by both oil addition and incubation pressure were significant ($p < 0.05$). OTUs affiliated with *Moritella* and *Psychromonas* made

strong contributions to the differences between pressure treatments (both $p < 0.01$). A large increase in the relative abundance of *Psychromonas* OTUs along with smaller increases in *Colwellia* and uncultured *Oceanospirillaceae* were observed at the RNA level at high pressure in comparison to atmospheric pressure. The genus *Psychromonas* comprised approximately 42 % and 16 % of total sequences in the HPO and HPNO treatments, respectively, whereas their relative abundance was below 1 % in all atmospheric pressure treatments at the RNA level. OTUs of *Psychromonas* showed 100 % sequence identity with *P. profunda* or *P. kaikoae*. In contrast, although *Moritella* OTUs were abundant in all PCB06 incubations, a lower relative abundance was observed with oil addition and at elevated pressure. *Moritella* contributed 26.6 %, 43 %, 76 %, and 85.6 % of total rRNA sequences in HPO, HPNO, APO, and APNO treatments, respectively. OTUs of *Moritella* were most closely related to *M. dasanesis* and *M. japonica* (both at 99 % similarity).

The LT4 represented the only shallow sediment in the experiment. Neither oil nor pressure treatment significantly impacted the community composition of incubations at the RNA or DNA level. An OTU of *Cobetia* was abundant in all treatments, and negatively correlated with oil addition. This OTU comprised 19.9 % and 28.9 % of total rRNA sequences in HPNO and APNO treatments as well as 14 % in both HPO and APO treatments. *Marinobacter* and *Planomicrobium* OTUs showed a substantially higher relative abundance at the RNA level. Other OTUs appeared to be impacted by incubation treatment. A *Marinobacter* OTU positively responded to both oil addition and elevated pressure. An *Alteromonas* OTU also positively responded to oil addition, but negatively responded to elevated pressure.

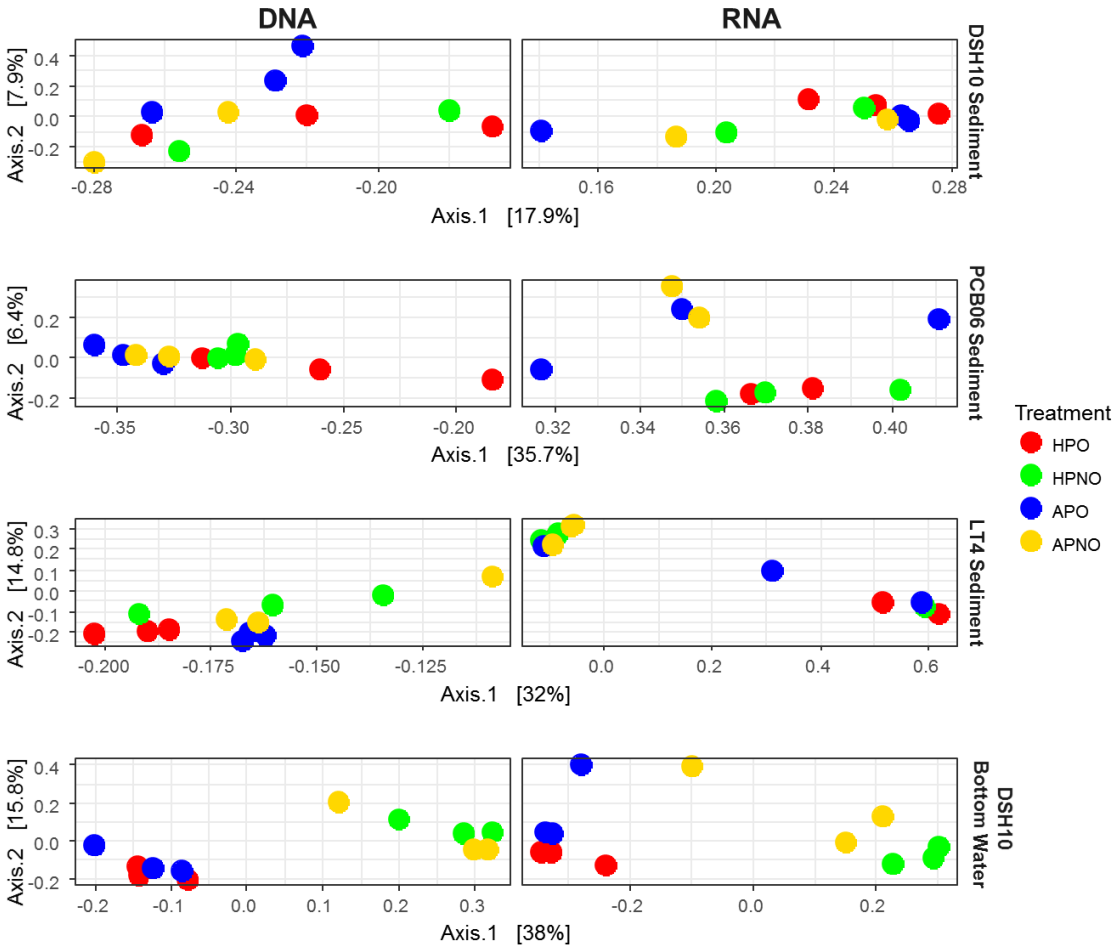


Figure 4-3. Variation in microbial community composition between incubation treatments. Left and right panels show microbial community composition from the sequencing of SSU rRNA genes in DNA and RNA extracts, respectively. Similarities between microbial communities are displayed as the Bray-Curtis distance metric on a PCoA plot.

In the DSH10 BW incubations, oil addition decreased taxonomic diversity regardless of pressure, mainly due to a predominance of two microbial groups, the genus of *Oleispiria* and a strain belongs to the C1-B045 clade of the *Gammaproteobacteria*. PERMANOVA suggested that oil addition represented a significant selective force on microbial communities and differences were not caused by dispersions within treatments. Pressure was a less significant factor and showed strong dispersion within treatment.

OTUs affiliated with uncultured *Oceanospirallaceae* and *Rhodobacteraceae* showed higher relative abundances of rRNA sequences at high pressure. *Methylophaga*, C1-B045, *Oleispira* OTUs were elevated in oil amended treatments. Other OTUs that were relatively low in abundance were also significantly affected by treatment, as shown in Supplementary Figure S4-2.

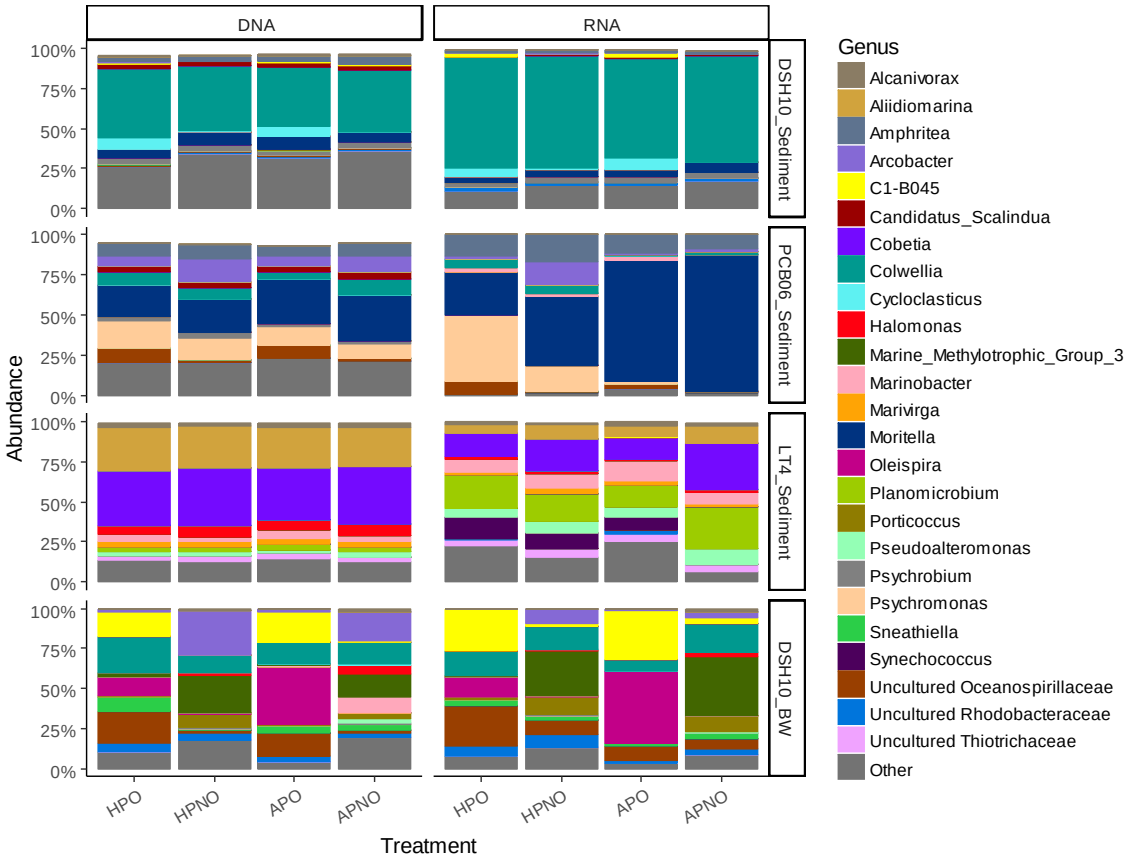


Figure 4-4. The relative abundance of microbial groups according to incubation treatment. Barplots represent the mean value from triplicate samples. Taxa are grouped at the genus level and relative abundance is calculated relative to total sequences.

4.5 Discussion

The majority of laboratory studies of hydrocarbon biodegradation have been performed under conditions that resemble the surface ocean, and relatively few studies have been conducted under high pressure and low temperature conditions that mimic deepsea conditions. This fundamental gap in the understanding of microbial hydrocarbon degradation is in contrast to the petroleum industry trend of increasing oil and gas production from ultradeep (>1500 m) wells and the risk of another deepsea oil well blowout in the Gulf of Mexico. Therefore, the goal of this study was to investigate how *in situ* microbial communities respond to oil contamination at pressures observed in the deepsea in comparison to atmospheric pressure.

Microbial activity has rarely been quantified at high pressures equivalent to those present in the deepsea and evidence is equivocal with regard to the impact of pressure on activity. In this study, while respiration in incubations of shallow marine sediments (LT4) from the continental shelf showed little impact of elevated pressure, activities measured in incubations of continental slope samples (DSH10, PCB06) were enhanced by 20 % to 80 % at the *in situ* pressure of 10 MPa. The pressure effect (PE) of between 1.3 and 1.8 observed here (PE = 1 – 3) is corroborated by rate measurements in previous studies of samples collected at comparable water depths [42,193,194], suggesting that hydrocarbon degrading-bacteria along with other functional guilds are adapted for optimal activity at elevated pressure. In contrast, previous work on hydrocarbon biodegradation at elevated pressure often observed a PE smaller than 1, suggesting inhibition of hydrocarbon degradation potential under elevated pressure. However, inconsistencies between studies may be explained by methodological constraints and the fact the previous studies did not adequately recapitulate in situ conditions. For example,

in studies conducted by Schwarz et al., while sediment samples were collected at 4,940 m water depth, the inoculum used for the experiments was enriched at atmospheric pressure and room temperature, despite the fact that these conditions have been shown to inhibit psychropiezophiles [84,184,185]. Many physiological studies conducted at high pressure have used pure cultures or inocula of micoorganisms obtained from environments exposed to atmospheric pressure. For example, Martina et al studied the effect of pressure on strains of *Rhodococcus* and *Sphingobium* that were originally isolated from surface seawater or a polluted stream[187]. Similarly, Scoma et al. also used *Alcanivorax* strains that were isolated from the surface seawater [186]. In addition, a slight inhibition of hydrocarbon degradation potential was reported in surface seawater at 15 MPa[85]. Since previous studies suggested that these surface originated microorganisms would be inhibited by elevated pressure [41], the decrease in activity was expected. As 10 MPa is considered to be a relatively mild pressure, no impact on microbial activity in LT4 was also anticipated. To obtain a true assessment of deepsea hydrocarbon degradation potential, studies must be conducted at temperature and pressure similar to the ambient condition, i.e. low temperature and high pressure for deepsea samples[189]. Moreover, it is logical to assume that ecosystems exposed to atmospheric pressure under normal conditions are less likely to contain microorganisms that are adapted to high pressure.

A number of physiological adaptations have been attributed to high hydrostatic pressures that occur in the deepsea. Grossi et al. isolated a strain of *Marinobacter hydrocarbonoclasticus* from deepsea sediment at 35 MPa[46]. Neither growth nor activity of this strain was inhibited to 35 MPa as determined by investigation of lipid composition. In the model piezophile, *Photobacterium profundum*, the relative abundance

of two different outer membrane proteins was regulated in response to elevated pressure [195]. The obligate psychropiezophile, *Profundimonas piezophila*, which does not grow at pressures lower than 20 MPa, was shown to survive at high pressure by modifying cell size and shape with elevated pressure[196]. Lastly, esterase activity associated deepsea microorganisms increased by 1.9 times at 20 MPa in comparison to atmospheric pressure, 0.1 MPa [49].

The results presented here suggest that the enhanced activity observed for hydrocarbon biodegradation may be extended to the decomposition of natural organic matter. Respiration in the no oil treatments was likely fueled by natural organic matter since no other carbon source was present [197]. Respiration rates measured in this study (90 to 435 µmol O_2/L sediment/day) are within an order of magnitude of previous rate determinations from pristine deepsea environments (~ 1000 µmol O_2/L sediment/day) [198]. This discrepancy may have been caused by substrate loss during the storage of our samples. It follows that no differences in respiration rate were observed in incubations of shallow sediments collected at LT4. Shallow sediment (~50 m water depth) would be expected to contain higher levels of labile carbon substrate and faster turnover rates [199]. This might lead to the preferential utilization of natural organic matter over oil.

Both oil and pressure acted as a disturbance that affected microbial community composition in our incubations of water and sediments from the deepsea. Shifts in microbial community composition were almost always more pronounced at RNA level than at DNA level. rRNA is used here as a proxy for the metabolically-active microbial groups as supported by previous work [200,201]. Thus, the results indicate that metabolically-active microbial populations respond more to oil and pressure, and studies at the DNA

level would be less likely to observe these effects. In incubations of shallow sediments (LT4) that are not normally exposed to elevated pressure, community composition was fairly consistent between all treatments at the RNA as well as DNA level. Higher alpha diversity was measured as Shannon Entropy but not as observed species, suggesting that the active microbial community was more evenly distributed, rather than more diverse, in LT4 incubations. No significant differences in beta diversity were observed between treatments in all sediment incubations, which may mean that both oil and pressure affected only a small portion of the microbial community. This may be due to the fact that oxygen supply was limiting in our experimental chambers. Total oxygen consumption comprised approximately 1 - 2 % of the total added oil carbon, assuming complete oxidation. In addition, as mentioned above, the pressure used (10 MPa) in the current study is considered to be relatively a mild elevation [186]. In some studies, the activity of unadapted microorganisms was not clearly affected until 40 to 50 MPa [48].

Taxa that were significantly impacted by oil and pressure treatments were identified by statistical analysis. Many known hydrocarbon-degrading bacteria are found within the class *Gammaproteobacteria* [153], and the majority of taxa detected in this study were from this group. For example, genus *Cycloclasticus,* which was first isolated from marine sediment in 1990s [202], is recognizes as an important obligate hydrocarbon degrader. This genus can utilize various polycyclic aromatic hydrocarbon compounds, including naphthalene, phenanthrene, anthracene, and toluene as the sole carbon source. During the DWH spill, *Cycloclasticus* was identified as a dominant member within deep oil plumes at a relative abundance of approximately 35 % [56]. Another group that was significantly affected by oil in this study was the C1-B045 clade of the family *Alteromonadaceae*. This

group was abundant and positively responded to oil addition in both DSH10 sediment and bottom water. Although little information is available, this clade was often found in the deep-sea environment [203], was more abundant at RNA level than DNA level [204], and was present in low abundance in oil contaminated sites [205]. OTUs of the marine methylotrophic group negatively responded to the oil addition in incubations of DSH10 bottom water. The closest cultured relative of these OTUs is *Methylophaga thiooxidans*, a sulfur-metabolizing bacterium that can oxidize dimethylsulfide to tetrathionate [206]. The genus *Methylophaga* was also detected in abundance in the deep oil plumes resulting from the DWH disaster [207], and transcriptomic analysis suggested that this group was associated with the terminal decomposition of dissolved organic matter [208].

OTUs affiliated with *Oleispiria antarctica* and uncharacterized members of the DWH *Oceanospirillales* clade showed contrasting responses to elevated pressure treatment in this study. The uncultured *Oceanospirillaceae* were more abundant at 10 MPa, while *Oleispira* was more abundant at 0.1 MPa. This suggests that the uncultured *Oceanospirillaceae* group is piezotolerant or piezophilic, since abundance at the RNA level was enhanced or not affected by elevated pressure. In fact, a species within the family *Oceanospirillaceae, Profundimonas piezophila*, was previously identified as a psychropiezophile, and the strain was demonstrated to be capable of hydrocarbon utilization [196]. By comparison, the decreased relative abundance of the genus *Oleispira* could be due to inhibition by elevated pressure or due to competition with the uncultured *Oceanospirillaceae*. *Oleispira antractica* was first described as an obligate alkane degrader in 2003 [209]. The model strain was obtained from an Antarctic coastal environment at 4 °C. The ambient conditions for this strain were permanently cold at atmospheric pressure,

which match our APO treatment. To our knowledge, *Oleispira* was not detected in high abundance in the GOM during the DWH discharge [210]. Interestingly, it is the closest cultured representative to the DWH *Oceanospirillales* (97 % similarity) [57], which was the dominant microbial community group in the oil plume at the early stage [8]. A cluster of uncultured *Oceanospirillales* that was 99 % to 100 % similar to the DWH *Oceanospirillales* was also enriched in our DSH10 bottom water incubations. Both *Oleispiria antarctica* and the DWH *Oceanospirillales* are considered alkane degraders [211], and could be competing for the same carbon source.

Other bacterial groups were affected by pressure in our incubations. In PCB06 sediments, two major groups, *Moritella* and *Psychromonas*, responded to pressure treatment. Although both of these strains were reported in hydrocarbon-degrading consortia [27,212], neither were confirmed as oil degraders. This is corroborated by our statistical analysis, which showed that the difference between oil and no oil treatments was mostly caused by variation within treatments in PCB06 sediment. The *Moritella* strain was in higher abundance at 0.1 MPa in comparison to 10 MPa. The most closely related cultured representatives, *M. marina* and *M. japonica*, showed different responses to pressure in previous work. *M. marina* was shown to be piezosensitive [213], while *M. japonica* was piezophilic [214]. Like *Moritella*, OTUs of our study affiliated with *Psychromonas* show nearly 100 % sequence similarity to multiple cultured representatives, including both piezophilic and piezosensitive strains.

Evidence from the current study indicates that the response of *Colwellia* to oil and pressure is strain-specific. *Colwellia* OTU #223 was found to decline in relative abundance at elevated pressure in DSH10 sediment incubations while other OTUs showed

a different response (Supplementary Figure 4-1 and 4-2). In bottom water incubations, two additional *Colwellia* OTUs, #229 and #255, were selected in the oil and no oil treatments, respectively, suggesting a distinct response to oil contamination. The genus *Colwellia* harbors diverse species that possess different metabolic capabilities. Through investigation of these OTU-level variations, the results point to ecological variation at the strain level. This strain-specific response is corroborated by previous studies by Kleindienst et al. in deep plumes generated by the DWH spill in the Gulf, which observed rare *Colwellia* oligotypes, suggesting adaptation to specific environmental niches [152].

In conclusion, the current study investigated the effects of high pressure on hydrocarbon-degradation potential and microbial community composition. In sediments and seawater of deepsea origin, elevated hydrocarbon degradation rates were observed at high pressure, which could be explained by either enhanced microbial activity or selection for pressure-adapted microbial populations. In contrast, hydrocarbon degradation rates in sediments from the shallow seafloor were not impacted by pressure treatment. The composition of metabolically-active microbial communities responded to oil addition and elevated pressure, suggesting these parameters preferentially select for certain microbial populations. This study is among the first to use *ex situ* incubations to determine the impacts of high pressure on the structure and function of indigenous microbial communities, which should aid in refining model predictions of oil fate to take into account the effects of pressure. Future studies should be conducted under ambient conditions and with samples originating from the deepsea in order to accurately predict hydrocarbon degradation potential.

CHAPTER 5. CONCLUSIONS

Understanding the influence of various oceanographic parameters on biodegradation processes is essential for predicting the trajectory of oil during/ after a spill as well as for improving emergency response efforts. Despite an extensive knowledge base, quantitative understanding is lacking, and we have not yet determined how environmental factors interact to regulate the fate and transport of spilled oil in the oceans. In part, this is due to methodological limitations and a lack of investigations at close to *in situ* conditions. Therefore, the objectives of this dissertation were to:

1. Quantify rates of petroleum hydrocarbon biodegradation in seawater and sediments under close to in situ conditions.

2. Investigate the interaction between various oceanographic parameters that limit biodegradation under conditions relevant to the DWH oil spill, including the application of dispersant chemicals as a response strategy.

3. Determine the fate of individual hydrocarbon compounds or compound classes under varying oceanographic or sedimentary conditions.

4. Link degradation pathways to microbial community dynamics and environmental functions.

Dispersant application is a primary emergency oil spill response strategy and yet the efficacy and unintended consequences of this approach to marine ecosystems remain controversial. To address these uncertainties, *ex situ* incubations were conducted to

quantify the impact of dispersant on petroleum hydrocarbon (HC) biodegradation rates and microbial community structure at close to *in situ* conditions in surface seawater. The results indicate that when applying dispersant to an oil slick, biodegradation may not be substantially enhanced unless sufficient mixing energy is provided. When the simulated slick was sufficiently dispersed, a higher rate of removal was observed for more recalcitrant hydrocarbon compounds (such as phenanthrene), suggesting that surface area available for microbial colonization is a primary factor limiting hydrocarbon degradation, and the application of dispersant will likely alleviate this constraint. While microbial growth and respiration were not substantially altered, RNA analysis revealed that dispersant application resulted in pronounced changes to the composition of metabolically active microbial communities. The quantitative increase in nitrogen-fixing members of the microbial community suggests a selection pressure for nitrogen fixation, likely indicating the robust response of the indigenous microbial communities to a readily biodegradable nitrogen-poor substrate. In order to improve model predictions and the bioremediation of dispersed oil during emergency response efforts, future study is warranted on the coupling of biodegradation to nitrogen fixation.

In microcosms of surficial seawater from representative geographical regions of major oil exploration (Beaufort Sea in the Arctic, northern and southern Gulf of Mexico, GOM), the microbial community response to oil contamination was shown to be site specific and dependent upon ambient conditions, temperature and nutrients. The activity, diversity, composition, and abundance of microbial communities were all strongly selected by site in seawater microcosms, suggesting adaptation to the local environment. The results indicate that the site of origin may override nutrient availability and temperature in

dictating hydrocarbon degradation potential. Surprisingly, the highest hydrocarbon degradation rates were observed in pristine polar waters. Thus, our results call into question the role of chronic oil pollution in the "priming" Gulf of Mexico waters for oil biodegradation. Known hydrocarbon-degraders of the *Gammaproteobacteria* dominated the microbial communities of all microcosms. While the putative psychrophilic group *Colwellia* dominated under cold conditions in polar seawater regardless of nutrient content, other groups (*Acinetobacter*, *Alcanivorax*, *Marinobacter*, *Alteromonas*) were important at mesophilic temperatures and communities responded at all sites to nutrient amendment. The genus *Acinetobacter* was observed in high abundance in the current study and field observation, but not found at low oil concentrations, suggesting different groups of microorganisms might be responsible for oil degradation under elevated oil concentration. It is important to note that this study represents only a snapshot of the likely spatiotemporal variation of the three sites, and should not be extrapolated to the entire geographical region. Future studies should incorporate direct measurements of HCs and focus on further verification of the complex interplay between environmental controls of biodegradation. The results indicate that both biodegradation activity and microbial community composition are dictated most strongly by the site of origin followed by nutrient availability and temperature.

Lastly, this dissertation investigated the effects of high pressure on hydrocarbon-degradation potential and microbial community composition. In sediments and seawater of deepsea origin, hydrocarbon degradation rates were elevated by 20 to 80 % at a pressure equivalent to that observed at 1000 m water depth, which could be explained by either enhanced microbial activity or selection for pressure-adapted microbial populations. In

contrast, hydrocarbon degradation rates in sediments from the shallow seafloor were not impacted by pressure treatment. The composition of metabolically-active microbial communities responded to oil addition and elevated pressure, suggesting that these parameters preferentially select for certain microbial populations. This study is among the first to use *ex situ* incubations to determine the impacts of high pressure on the structure and function of indigenous microbial communities, which should aid in refining model predictions of oil fate to take into account the effects of pressure.

In summary, this dissertation work tested the hydrocarbon biodegradation potential under the influence of various oceanographic controls, including mixing, dispersant application, temperature, major nutrients, and pressure. The results indicate that when applying dispersant to an oil slick, biodegradation may not be substantially enhanced unless sufficient mixing energy is provided. The activity, diversity, composition, and abundance of microbial communities were all strongly selected by site in microcosms of oil-contaminated seawater, suggesting adaptation to the local environment. The results indicate that the site of origin may override nutrient availability and temperature in dictating hydrocarbon degradation potential. However, dissertation results did not support the hypothesis that the Gulf of Mexico is inordinately "primed" for oil biodegradation due to extensive exposure to natural seeps. Evidence for microbial community adaptation to ambient conditions suggested the degradation experiments should be conducted at the environmentally-relevant conditions. This dissertation delivers rate measurements that can be employed immediately to improve numerical models to predict the fate and transport of spilled oil in the oceans. In addition, the dissertation research provides critical insights into the complex interplay of oceanographic parameters that limit hydrocarbon degradation.

5.1 Future Research

As oil exploration activities are moving towards nontraditional regions, such as the Arctic and ultradeep waters, the potential risk of oil contamination is rising for pristine ecosystems. It is critical to assess the environmental risk and produce contingency plans for oil spills. Comprising the primary means by which spilled oil is eliminated from the environment, microbially-mediated degradation processes need to be further investigated in order to better estimate the trajectory of oil in the ocean.

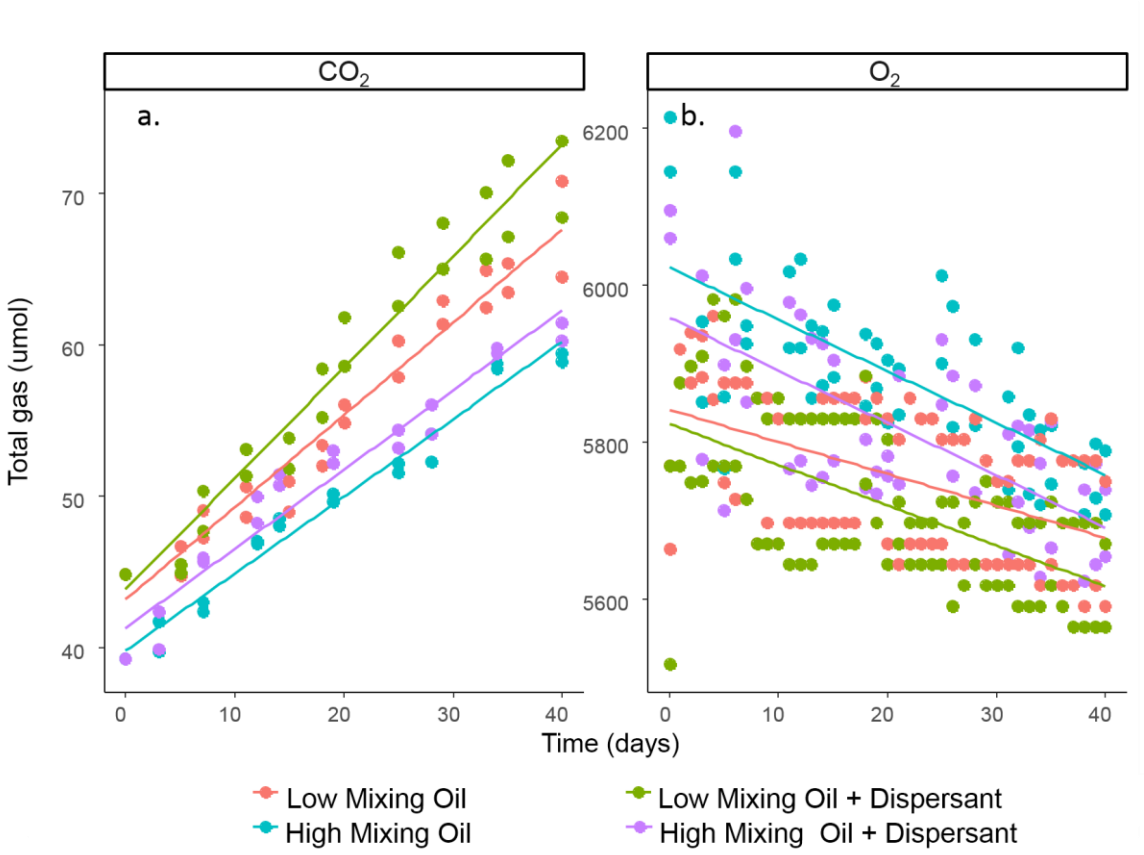
In this dissertation, results demonstrated that only a portion of the oil was mineralized through biodegradation, leaving a substantial amount of intermediates that were not amenable by gas chromatography [104,215]. Further studies are needed on the formation and fate of these intermediate compounds.

In chapter 2, results demonstrated the importance of the experimental design in dispersant studies. It is important to realize that liquid hydrocarbons on the sea surface are either dispersed or retained as a slick shortly after a spill. This suggests that the determination of degradation potentials in separate laboratory incubations of dissolved oil (WAF) and dispersed oil (CEWAF) treatments may not adequately reflect the efficacy of dispersant application on the sea surface. Moreover, dispersed oil is usually rapidly diluted the sea surface within days after a spill, which demands *ex situ* incubations to be conducted at low oil concentration to simulate the *in situ* condition. Lastly, through molecular techniques, the results revealed that nitrogen fixation was stimulated during degradation, possibly as a response to nutrient limitation. Future studies should investigate the coupling between biodegradation and nitrogen fixation in the environment.

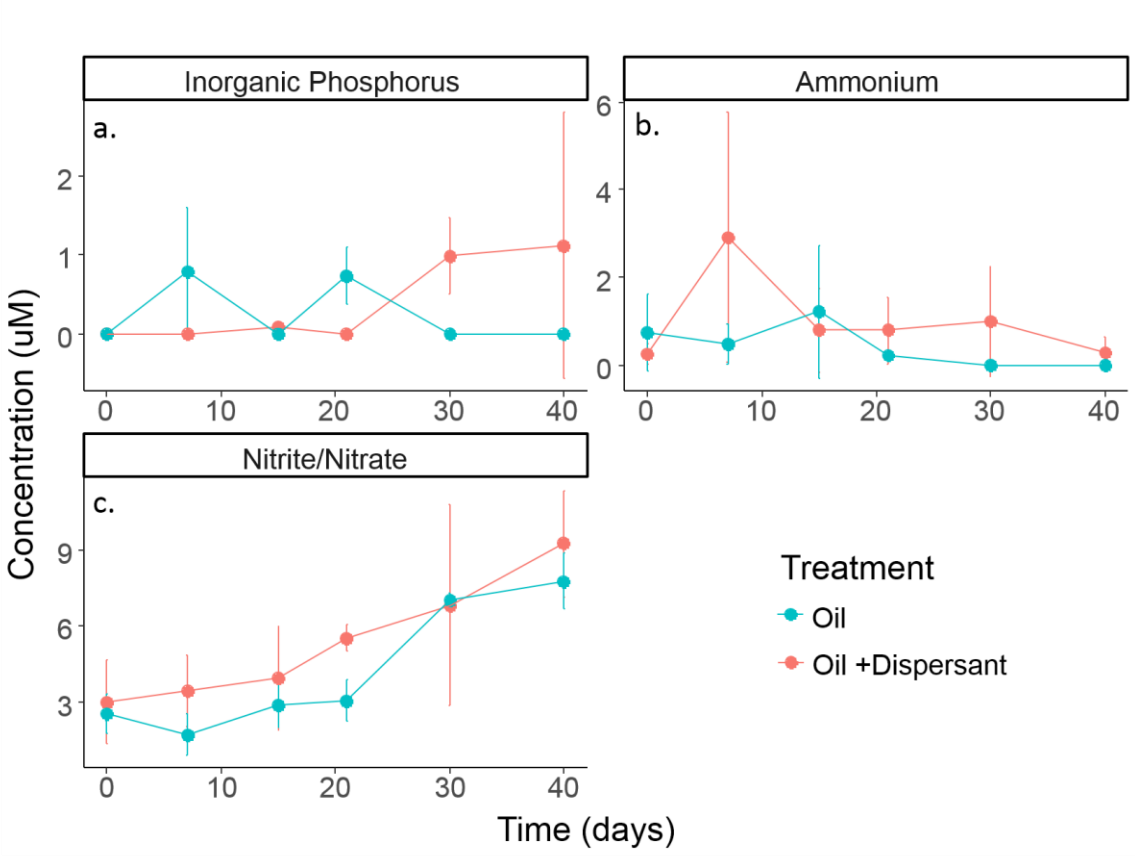
In chapter 3, microbial adaptation was suggested to be the primary factor in controlling hydrocarbon degradation potential, as biodegradation rates and the composition of indigenous microbial communities were shown to covary with *in situ* environmental conditions. By using respiration as a proxy for biodegradation, we observed the highest oil degradation rates within pristine Arctic surface seawater in comparison to seawater from the northern and southern GOM. Future investigations should incorporate direct measurements of HCs to further confirm the elevated oil degradation potentials observed in the Arctic. In addition, since the observed spatiotemporal variation was based on a limited number of sites, additional geographical locations should be tested for further evidence of the "priming" hypothesis.

Chapter 4 focused on the effects of pressure on hydrocarbon degradation potential. The results demonstrated *in situ* pressure could enhance hydrocarbon degradation in seawater and sediments from the deepsea. Observations indicate that future studies should be conducted at ambient pressure in order to accurately simulate processes in the deepsea. Furthermore, recent studies have shown that the decompression of deepsea samples may lead to decreased microbial activity even in incubations conducted at *in situ* pressure [216]. Although pressure-retaining samplers are rare and expensive, future research should employ these devices to avoid underestimation of microbial activity.

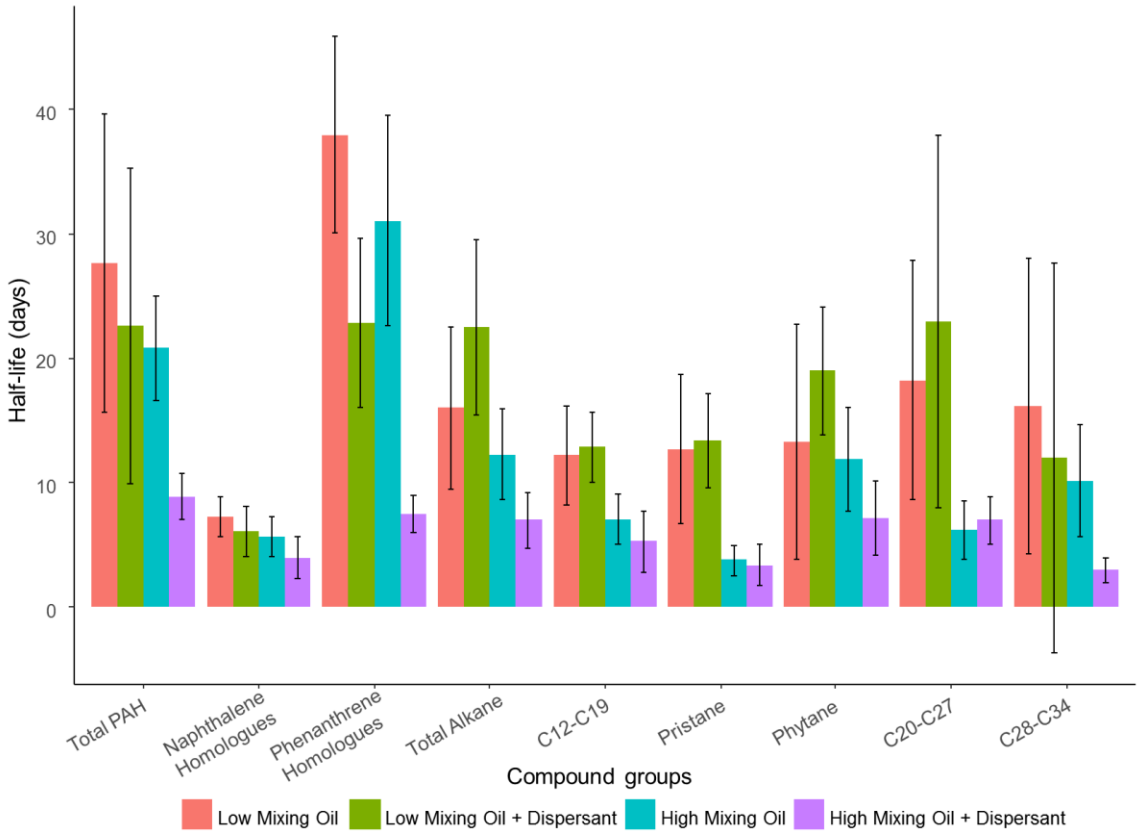
APPENDIX A. SUPPLEMENTARY FIGURES FOR CHAPTER TWO



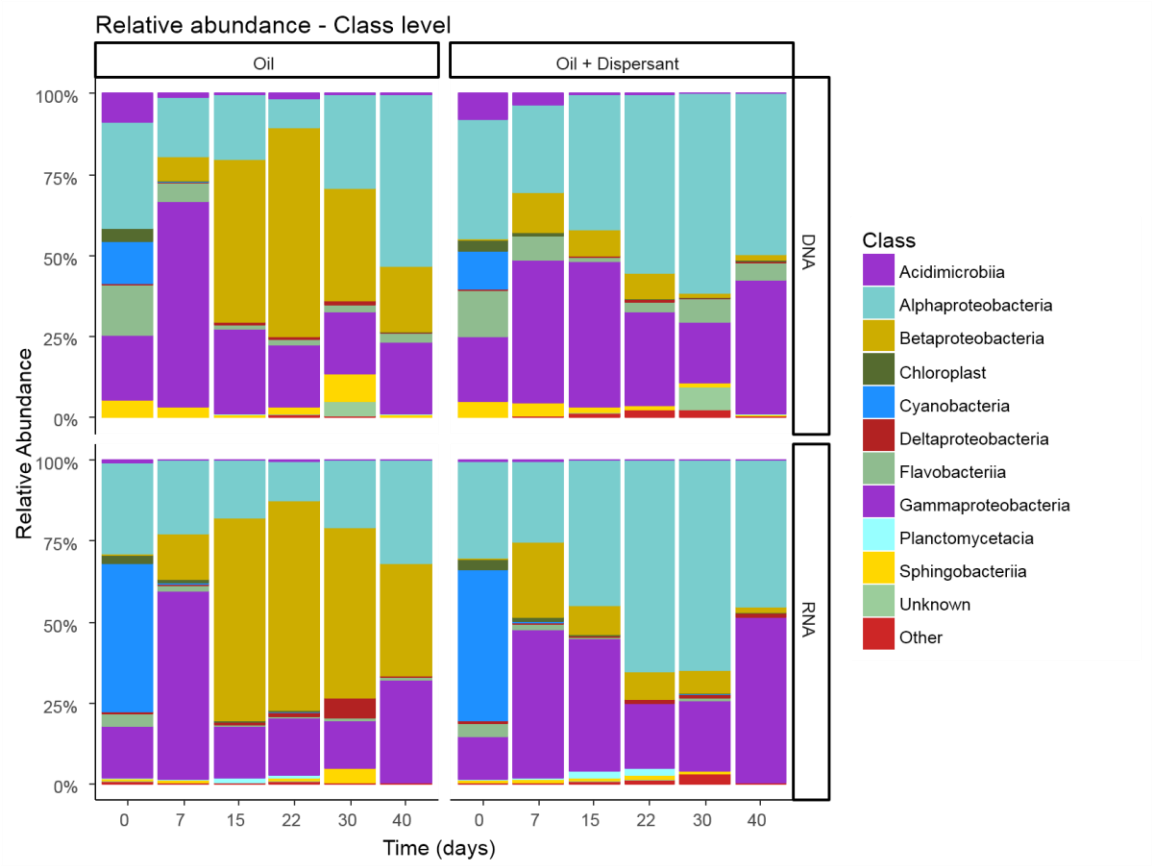
Supplementary Figure 2- 1. Microbial respiration as determined by carbon dioxide accumulation and oxygen consumption in seawater microcosms. The left and right panels show total carbon dioxide and total oxygen in the microcosms, respectively. Lines are fitted linear regressions.



Supplementary Figure 2- 2. Concentrations of major inorganic nutrients in the seawater microcosms. Values shown are averages from four replicate measurements. Error bars are standard deviations.

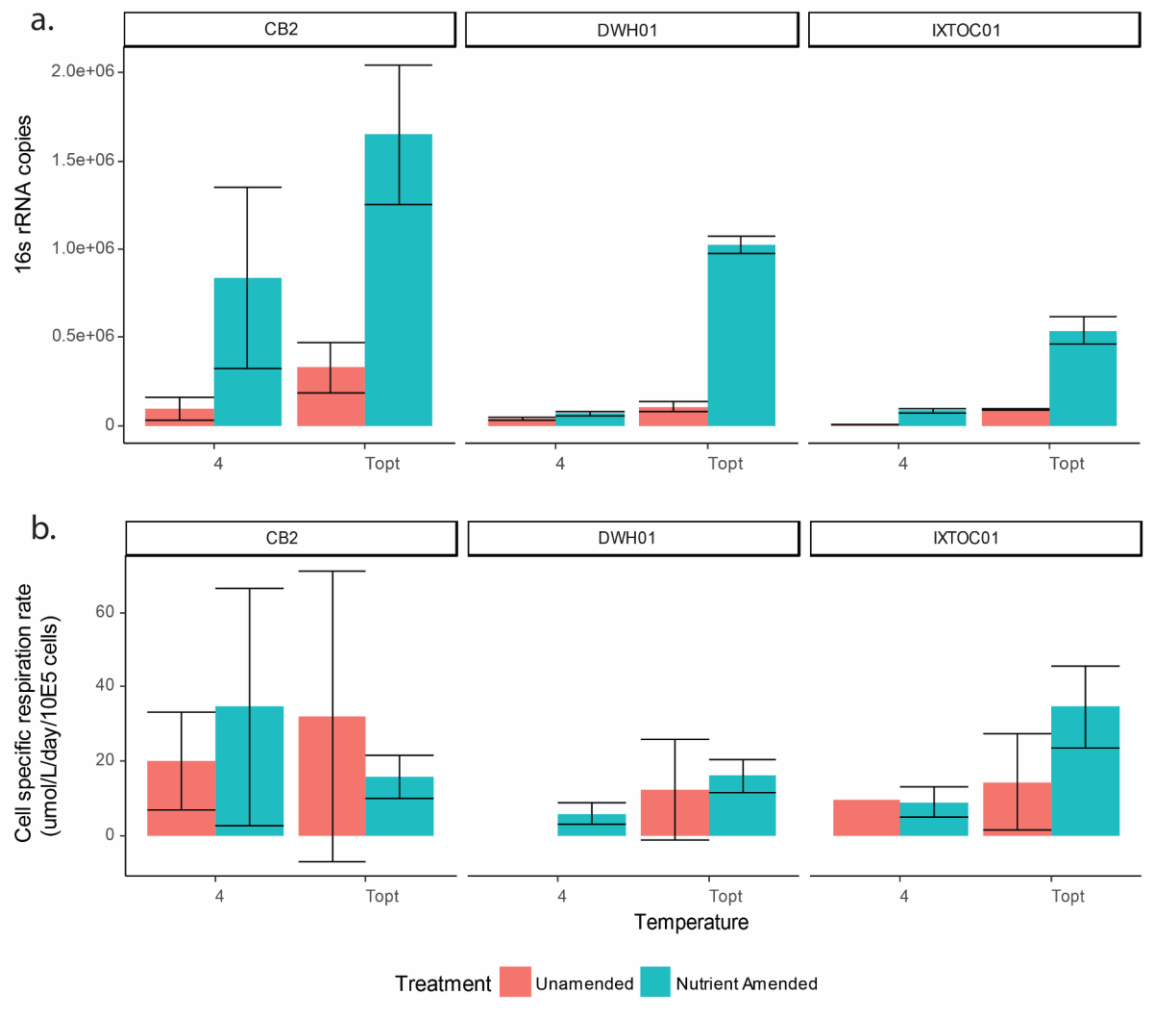


Supplementary Figure 2- 3. First order kinetic half-lives for hydrocarbon removal. The half-lives are calculated using transformed data to fit a first order kinetic equation and error bars represent the standard deviation of the fitted curve. This method is less accurate for labile compounds in the high mixing dispersant treatment due to their potential rapid removal before the first measurement.

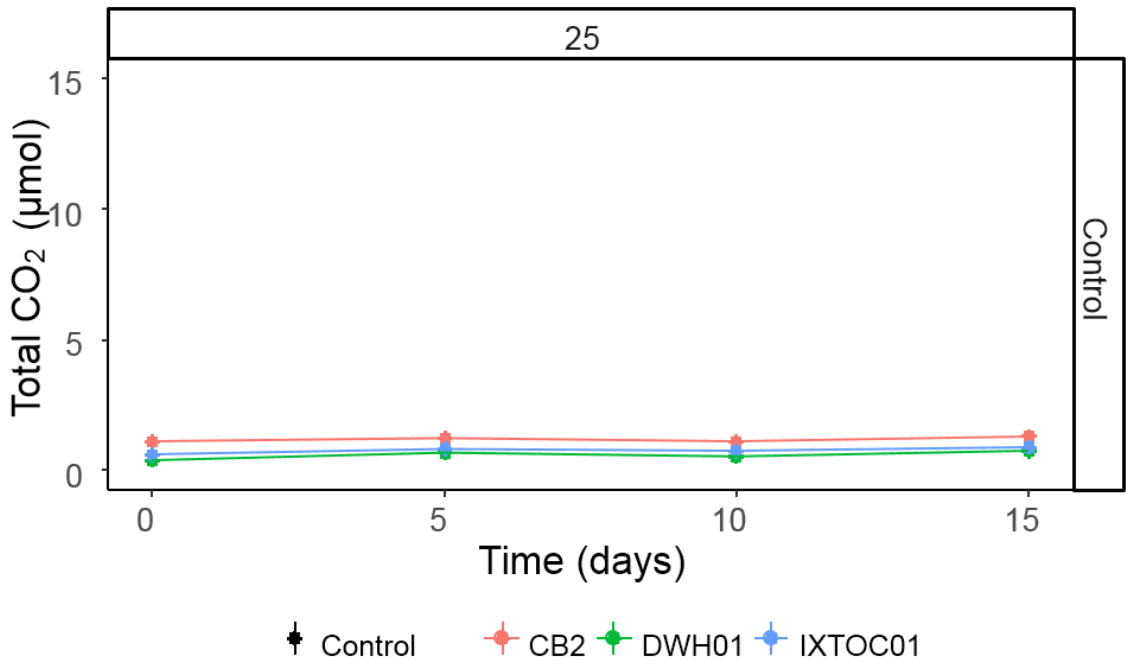


Supplementary Figure 2- 4. The relative abundance of microbial groups at the phylum to class level with incubation time and treatment. Barplots show mean values of duplicated samples. Taxa are grouped at the class level and relative abundance is calculated relative to total sequences retrieved for each class.

APPENDIX B. SUPPLEMENTARY FIGURES FOR CHAPTER THREE

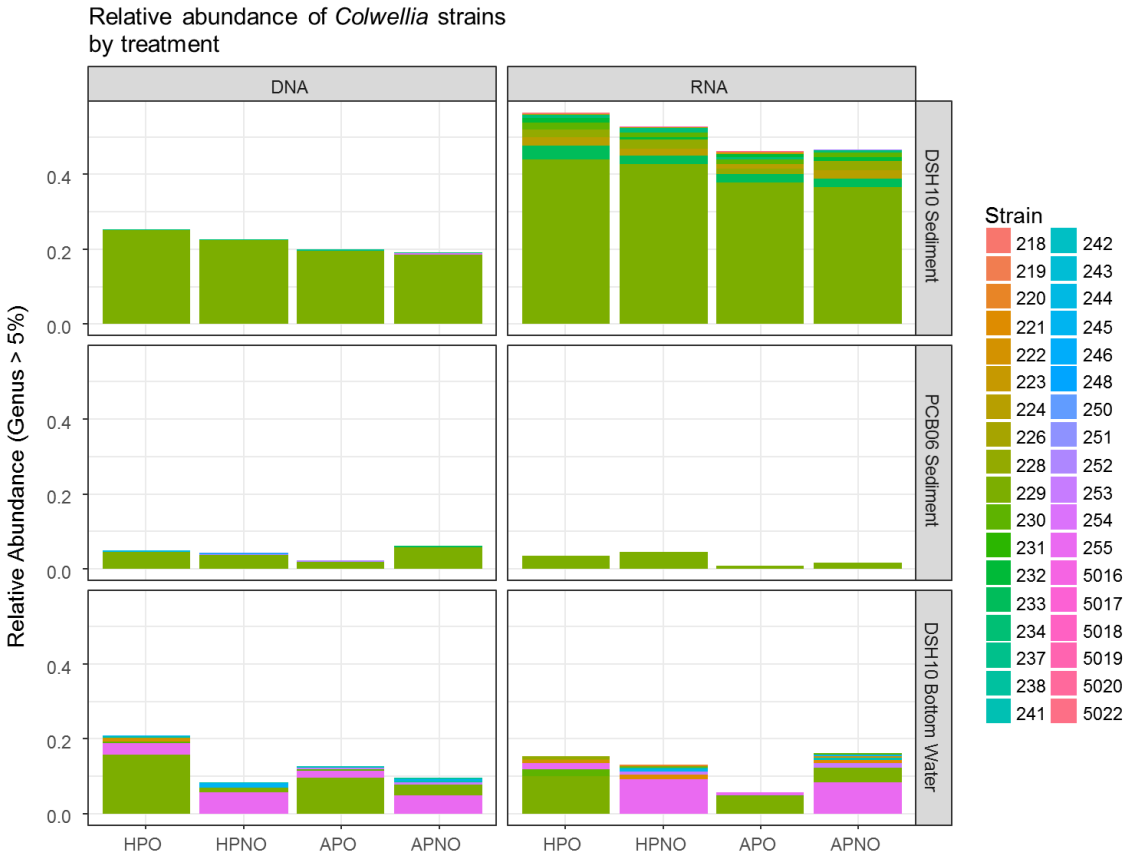


Supplementary Figure 3- 1. SSU rRNA gene abundance and cell specific respiration rates at 4 °C and T_{opt} (30 °C for CB2 and 38 °C for DWH01 and IXTOC01). Barplots show mean value from triplicate measurements for each of the triplicate samples. Error bars are standard deviations.

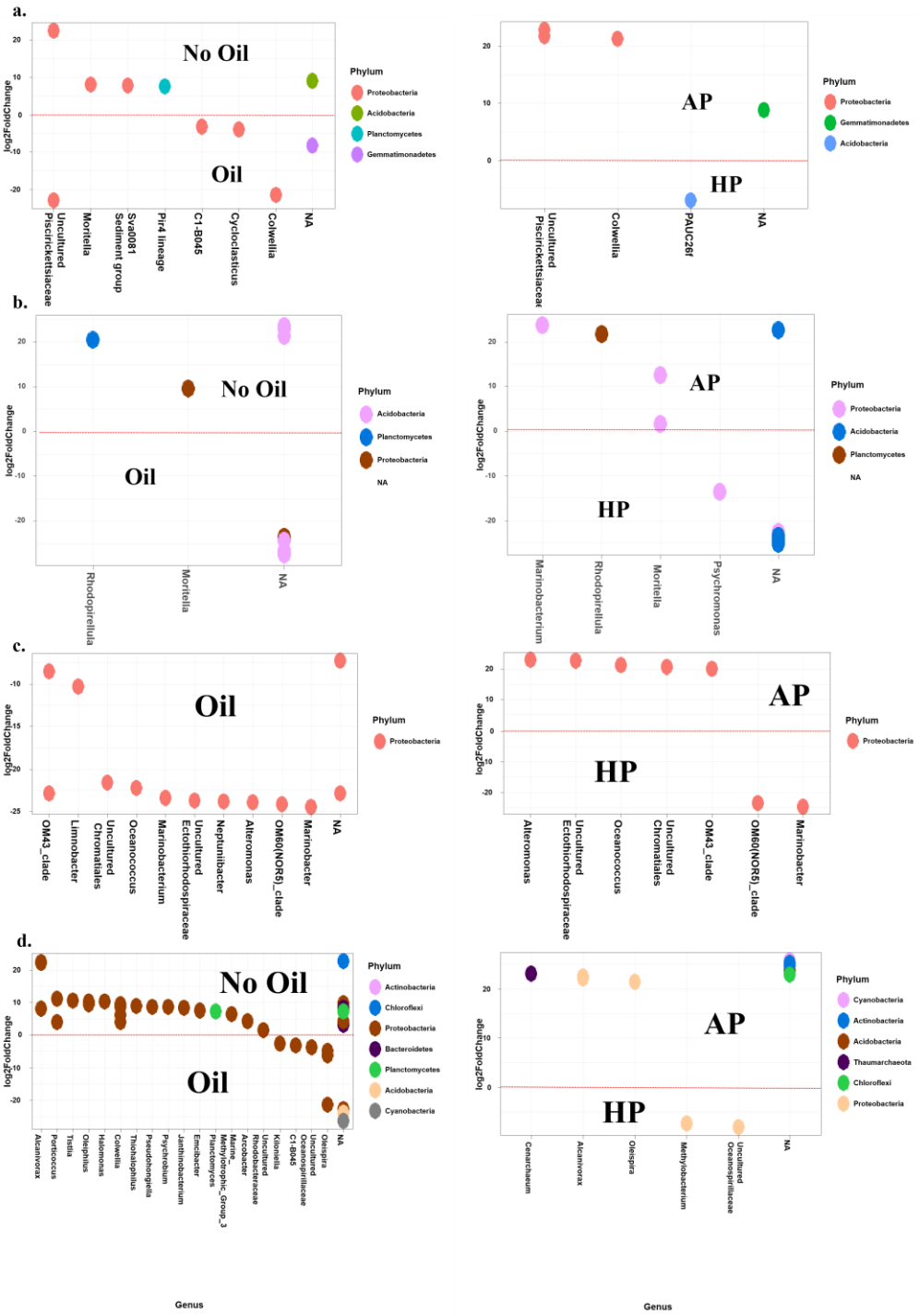


Supplementary Figure 3- 2. CO_2 accumulation in no oil controls. Scatter plots show average values from triplicate samples. Error bars are standard deviations.

APPENDIX C. SUPPLEMENTARY FIGURES FOR CHAPTER FOUR



Supplementary Figure 4- 1. The relative abundance of members of the genus *Colwellia* at the strain level within each treatment. Barplots show mean values from triplicate samples. Relative abundance is calculated relative to total sequences retrieved.



Supplementary Figure 4- 2. The impact of treatment on individual taxa. Panels on the left and right show taxa affected by oil addition and by pressure, respectively. The four rows of panels from top to bottom represent incubations of the following samples: DSH10 sediment, PCB06 sediment, LT4 sediment, and DSH10 bottom water.

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